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VOL. LII JULY–AUGUST, 1960 NO. 4

FUNGI OF NEW MEXICO

HAROLD H. KUEHN

Practically nothing is known regarding the mycological flora of New Mexico with the possible exception of certain groups of Gasteromycetes. Therefore, it seems worthwhile to summarize the work of previous investigators and offer some additional contributions.

The earliest published report concerned with New Mexican fungi was made by Cockerell (**1904**) in which he listed 46 species, including 31 species of Uredinales, 7 species of other Basidiomycetes, 5 species of Ascomycetes, 2 species of Moniliales and one myxomycete. This initial report of fungi from the state was followed by a list of 25 species of Myxomycetes which Macbride (**1905**) collected in one season. Wooton and Standley collected occasional fungal specimens during their field studies of higher plants of the state (**1915**), with the result that Standley (**1916**) was able to make a contribution to the mycological knowledge of New Mexico. Standley stated that his collections were especially concerned with parasitic fungi, and furthermore, these collections were limited to 4 or 5 northern counties. Since Standley admittedly was not a mycologist his specimens were determined by specialists in the various groups. Of the 211 species reported by Standley all were parasites with the exception of 14 Agaricales and 8 Lycoperdales. The following list shows the distribution by orders of Standley's collections: Chytridiales 1 species, Peronosporales 9, Sphaeriales 21, Pezizales 2, Uredinales 113, Ustilaginales 21, Agaricales 14, Lycoperdales 8, Sphaeropsidales 12, Moniliales 10. After studying some of Standley's collections from New Mexico, Fairman (**1918**) described many ascomycetes and deuteromycetes found on parts of higher plants.

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According to Standley, several mycologists—"S. M. Tracy, F. S. Earle, David Griffiths, G. G. Hedgcock, E. W. D. Holway and perhaps others"—had collected previously in New Mexico. Also, in 1914 Arthur and Fromme collected Uredinales within the state. Included in the 211 species reported by Standley were some collected by Wooton, Cockerell and Arthur. Standley followed with a second paper (**1918**) in which 45 species of Uredinales and 4 of Ustilaginales were reported, and here Standley gave further information regarding scattered reports of New Mexican rusts by other investigators.

The investigator who contributed most to our knowledge of fungi from New Mexico was William Henry Long, whose publications span 46 years. His studies which pertained to fungi from New Mexico include those discussing rusts (Long, **1903**, **1916b**, **1917a**; Long and Harsch, **1918**), those concerned with *Polyporus* or wood diseases (Long, **1916a**, **1917b**, **1918**, **1930**, **1945**), one dealing with *Coprinus* (Long and Miller, **1945**), and those treating the Gasteromycetes (Long, **1942a**, **1942b**, **1943**, **1944**, **1946a**, **1946b**, **1946c**, **1947**; Long and Plunkett, **1940**; Long and Stouffer, **1941**, **1943a**, **1943b**, **1946**, **1948a**, **1948b**, **1949**). Other references to fungi from New Mexico include White (**1901**), Hedgcock and Long (**1912**), Hedgcock and Hunt (**1916**), Arthur (**1934**) and Fischer (**1953**).

The writer was privileged to spend several years in New Mexico from 1954 through 1957, during which period he had occasion to collect and study fungi from time to time. However, the collections were haphazard and sporadic, and by no means should be regarded as indicative of the sum total of fungi to be found in the regions in question. Because of the writer's interests the fungi collected and examined were almost exclusively soil fungi and water-molds, although other fungi, particularly Lycoperdales, were found to be quite prevalent throughout the mountains of New Mexico.

MATERIALS AND METHODS

Water samples were collected from some bodies of water located in the mountainous portions of San Miguel and Taos counties of northern New Mexico from September through December, 1954. The water samples were collected in sterile screw-cap test tubes and kept at the existing temperature until brought into the laboratory. The water was placed in sterile Petri dishes and baited with two or three split hemp seeds. Transfers were made until pure cultures were obtained, at which time each isolate was studied and identified, using Coker (**1923**) and Harvey (**1942**).

Several fish dying of a fungal parasite were obtained from the Red River fish hatchery, Questa, New Mexico. This parasite subsequently proved to be *Saprolegnia parasitica*.

Soil samples were taken from dry desert soils of the Tularosa Basin, Otero county, in southern New Mexico, during 1956–1957. This study of soil fungi of desert soils was confined primarily to those found in the nitrogen-deficient gypsum sand dunes of the White Sands, part of which form White Sands National Monument, located 15 miles west of Alamogordo, New Mexico. Since this region differs considerably from the usual type of substrate, it is considered apropos to give a brief description of the area at this time.

TABLE I

WATER-MOLDS ISOLATED FROM 56 SAMPLES

Fungus	Location
Saprolegnia delica Coker	1. Gallinas reservoir, Montezuma, 6700 ft
Saprolegnia ferax (Gruith.) Thuret	1. Gallinas river, Montezuma Hot Springs, 6700 ft
	2. Pond near Camp Luna, Las Vegas, 6700 ft
	3. Gallinas river, at 4H-club camp, 8000 ft
	4. Gallinas reservoir, Montezuma, 6700 ft
Saprolegnia parasitica Coker	1. On trout, Red River Fish Hatchery, 7500 ft
Saprolegnia sp. (non-fruiting)	1. Ponds, Evergreen Valley, Hollinger Canyon, 8500 ft
	2. Gallinas reservoir, Montezuma, 6700 ft
Achyla americana Humphrey	1. Rio Pueblo, between Tres Ritos and Holman Pass, 8500 ft
Achyla sp. (non-fruiting)	1. Gallinas reservoir, Montezuma, 6700 ft
Dictyuchus sp. (non-fruiting)	1. Gallinas river, Montezuma Hot Springs, 6700 ft

Lying at an altitude of 4250 ft, this area of 224 square miles, 28 miles long and about 10 miles wide, is composed of gypsum dunes of about 96–97% calcium sulfate (Coville and MacDougal, **1903**; Byers, **1936**). Most of the dunes drift about 9 inches per year, with the wind constantly removing the lighter particles of humus, etc. This area is occupied by dunes up to 40 ft high and narrow wind-formed flats interspersed among the dunes. The water table in the flats is about 2–3 feet below the surface, while the soil pH is 7.4 (Byers, **1936**). According to Emerson (**1935**) numerous tests by soil chemists have shown traces of nitrogen in only one instance. Potassium and phosphorus are present in minute amounts (Coville and MacDougal, **1903**).

The soil samples were collected in sterile screw-cap test tubes which were pushed into the sand to a certain desired depth. Soil samples con-

TABLE II

FUNGI ISOLATED FROM TULAROSA BASIN SOILS OTHER THAN GYPSUM SAND DUNES

Alternaria humicola Oudemans
Aspergillus niger van Tieghem
Aspergillus ochraceus Wilhelm
Aspergillus sydowi (Bain. and Sart.) Thom and Church
Chaetomium globosum Kunze
Fusarium sp. 1
Helminthosporium sp.
Hormodendrum pallidum Oudemans
Phoma sp.
Spicaria violacea Abbott

sidered to be of surface layers were limited to the upper ½ inch of sand. Other collections were made of sand at a depth of 2–5 inches (referred to as "subsoil" in TABLE III) in order to determine whether there was a correlation of fungus species with depth. When it was necessary to

TABLE III

FUNGI ISOLATED FROM THE WHITE SANDS

Fungus	Sand Stratum	
	Surface	Subsoil
Alternaria geophila Daszewska		+
Alternaria humicola Oudemans	+	+
Alternaria sp. 1	+	+
Alternaria sp. 2	+	+
Aspergillus caespitosus Raper and Thom	+	
Aspergillus flavus Link	+	+
Aspergillus fumigatus Fres.	+	+
Aspergillus luchuensis Inui		+
Aspergillus nidulans var. *alba* Yuill	+	+
Aspergillus niger van Tieghem	+	+
Aspergillus ochraceus Wilhelm	+	
Aspergillus restrictus G. Smith		+
Aspergillus sulphureus (Fres.) Thom and Church		+
Aspergillus sydowi (Bain. and Sart.) Thom and Church	+	
Aspergillus ustus (Bain.) Thom and Church	+	+
Aspergillus versicolor (Vuill.) Tiraboschi	+	+
Cephalosporium curtipes Sacc.	+	+
Curvularia geniculata (Tracy and Earle) Boedijn		+
Curvularia tetramera (McKinney) Boedijn	+	
Emericella nidulans (Eidam) Vuill.	+	+
Emericella rugulosa (Thom and Raper) Benjamin	+	+
Emericella quadrilineata (Thom and Raper) Benjamin	+	+
Epicoccum nigrum Link		+
Eurotium chevalieri Mangin		+
Fusarium sp. 1	+	
Fusarium sp. 2	+	+
Gliobotrys alboviridis von Höhnel		+
Gliocladium catenulatum Gilman and Abbott	+	
Helminthosporium sp.	+	+
Hormiscium sp.	+	+
Hormodendrum cladosporioides (Fres.) Sacc.	+	
Hormodendrum pallidum Oudemans	+	

TABLE III (CONTINUED)

Fungus	Sand Stratum	
	Surface	Subsoil
Hormodendrum resinae Lindau		+
Hormodendrum sp. 1	+	
Hormodendrum sp. 2	+	
Hormodendrum sp. 3	+	
Hormodendrum sp. 4	+	
Memnoniella echinata (Riv.) Galloway		+
Myrothecium verrucaria (Alb. and Schw.) Ditmar	+	+
Nigrospora sphaerica (Sacc.) Mason	+	
Penicillium adametzi Zaleski		+
Penicillium citrinum Thom		+
Penicillium cyaneo-fulvum Biourge		+
Penicillium decumbens Thom		+
Penicillium expansum Link		+
Penicillium lanosum Westling	+	
Penicillium lilacinum Thom	+	+
Penicillium martensii Biourge		+
Penicillium nigricans (Bain.) Thom	+	
Penicillium notatum Westling	+	+
Penicillium paxilli Bain.		+
Penicillium raistrickii Smith		+
Penicillium simplicissimum (Oudemans) Thom		+
Penicillium steckii Zaleski	+	
Penicillium sp. 1	+	
Penicillium sp. 2		+
Penicillium sp. 3	+	
Phoma sp.	+	+
Rhizopus arrhizus Fischer	+	
Stachybotrys atra Corda		+
Stemphylium verruculosum Zimmermann	+	+
Torula allii (Harz) Sacc.	+	
Unknown 1 (Moniliaceae)		+
Unknown 2 (Dematiaceae)		+
Unknown 3 (Sphaeropsidaceae)	+	

dig down several inches into the sand, a sterile trowel was used for this purpose. The various samples were screened within a day after collection by soil dilutions and direct inoculation of the soil onto plates of hay-infusion agar, which was prepared according to Thom and Raper (**1945**, p. 35). To avoid loss of slow-growing fungi all the plates were examined once a day, using a binocular dissecting microscope. The plates were retained and examined daily for about one month, after which they were discarded. Species of *Penicillium, Aspergillus, Spicaria* and *Gliocladium* were subcultured to Czapek's agar as an aid in identification. Other fungi were subcultured to potato-dextrose agar for examination.

RESULTS

A total of 77 species representing 33 genera was discovered. The distribution among the groups of fungi was as follows: Myxomycetes, 2; Saprolegniaceae, 7; Mucorales, 2; Ascomycetes, 6; Deuteromycetes, 60.

In addition to these fungi many species of *Streptomyces* were encountered during isolation procedures from the gypsum sands. TABLES I, II and III list the fungi which were isolated and the area from which they were taken.

DISCUSSION

As far as the writer has been able to ascertain no previous studies have been undertaken of soil- or water-molds from New Mexico. Harvey (**1942**) did not make any collections within New Mexico; however, he achieved no success in attempts to isolate species of Saprolegniaceae from 83 samples of soils and water collected in Utah and Arizona. He initially postulated a correlation between altitude and lack of water-molds, since he found few such fungi at elevations above 5000 ft in extensive collections throughout the west. Later collections by Harvey in California, however, produced water-molds from altitudes up to and exceeding 9000 ft. The water-molds isolated in the present study might all be regarded as from high elevations, having been found from 6700 ft to 8500 ft. While it might be true, as Harvey suggested, that water-molds are not as plentiful at elevations above 5000 ft, nevertheless it is quite evident that the water-mold flora at such elevations is certainly more prevalent than initially suspected.

Shields (**1951**, **1954**) stated that because of the extremely low carbohydrate content of the gypsum sands a thriving saprophytic, nitrogen-fixing flora has developed. She further noted the occurrence of "miscellaneous fungi which may have nitrogen fixing potentialities." In a review of nitrogen sources of plants, Shields (**1953**) refers to the following reports of nitrogen fixation by fungi: *Phoma radicis, Phoma radicis callunae* and *P. betae* have been reported to fix nitrogen (Bose, **1943**), while according to Frei (**1942**), Fulmer and Christensen (**1925**) and Schanderl (**1942**) small amounts of nitrogen are fixed by *Endomyces vernalis, Penicillium glaucum, Saccharomyces, Willia, Pichia* and *Torula* species as well as by *Mycoderma bispora*. In view of these reports the above fungi were sought for especially during the course of this investigation. It is interesting to note that *Torula allii, Penicillium expansum* and an unidentified species of *Phoma* were isolated from the nitrogen-deficient gypsum sands. According to Raper and Thom (**1949**, p. 522), *Penicillium glaucum* is not a definite specific entity, but in many instances probably referred to *P. expansum*.

About 60 species of angiosperms grow sparsely in the peripheral areas of the dunes. According to Schaffner (**1948**) 28 species of dicotyledons which grow in the surrounding area are lacking in the sand dunes, while several plants which grow within the dune area are not found in the

surrounding desert plains. Actually, 5 species which grow in White Sands are considered by Wooton and Standley (**1915**) to be specific indicators of gypsum. When the present study of soil fungi from White Sands was initiated, it was hoped that certain species would be isolated which could be regarded as indicators of gypsum similar to the 5 angiosperm species already referred to. To my knowledge, however, none of the isolated fungi could be considered to be indicators. The fungus group which was most often encountered was the *Aspergillus nidulans* group. The 3 species, *A. nidulans* (*Emericella nidulans*), *A. rugulosus* (*E. rugulosa*) and *A. quadrilineatus* (*E. quadrilineata*) were the most frequently isolated fungi, and for this reason might be tentatively regarded as characteristic of the White Sands were it not for their reportedly frequent occurrence in dry, warm soils of Texas and Arizona (Thom and Raper, **1945**, p. 170). The unidentified species of *Hormiscium* was frequently encountered also. In this study, the only species which were isolated from desert soils but not from the White Sands were *Chaetomium globosum* Kunze and *Spicaria violacea* Abbott.

Mucorales (with one exception) were conspicuous by their absence from soils of the Tularosa Basin. It might be mentioned here that a species of *Pilobolus* was discovered on deer dung collected October 28, 1954, in Evergreen Valley, west of Las Vegas. This species was tentatively identified as *P. crystallinus* (Wiggers) Tode.

The writer made several collecting trips in search of Myxomycetes in the Sangre de Cristo Mountains of northern New Mexico and in the Sacramento Mountains of the southern portion of the state. The result was the collection of two species, both from northern New Mexico. *Trichia scabra* Rost. was collected in Evergreen Valley, San Miguel county, while *Ceratiomyxa fructiculosa* (Mull.) Macbr. was located several times along the Rio Pueblo on state route 3 near Tres Ritos, Taos county. Macbride (**1905**) reported neither of these species in his collections.

The fungi presented in this paper are not the first to be reported from the White Sands. Long found several species of Gasteromycetes in the dune area and his collections are summarized here: 83 specimens representing 3 species of *Tylostoma* (**1944**), 148 specimens of *Chlamydopus meyenianus* (Klotzsch) Lloyd (Long and Stouffer, **1946**) and 4 plants representing 3 species of *Geaster* (Long and Stouffer, **1948a**).

SUMMARY

A brief history is presented of mycological collections in New Mexico. Isolation of water-molds from some bodies of water in the Sangre de

Cristo Mountains of northern New Mexico and isolation of fungi from the desert soils and gypsum sands of the Tularosa Basin in southern New Mexico resulted in reports of 77 species of fungi. Included were 7 species of Saprolegniaceae from elevations exceeding 6700 ft, and 60 species of Fungi Imperfecti.

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KANSAS AEROMYCOLOGY V: PENICILLIUM AND ASPERGILLUS [1]

C. L. Kramer, S. M. Pady and C. T. Rogerson [2]

(with 3 figures)

INTRODUCTION

Previous papers (**6, 7, 8, 19**) have reported other aspects of a two-year study (9–16–56 to 8–30–58) of fungus populations of the air in Kansas. The project was designed to study fungus spores using silicone slides as well as colonies that could be obtained by exposing petri plates of nutrient media. However, in the case of the two genera *Penicillium* and *Aspergillus,* treated in detail here, this could not be done since their spores are indistinguishable from one another and from many other genera. For this reason, all information in the following discussion is based on colony studies only.

The standard medium used was rose bengal-streptomycin (RBS) agar since earlier work had shown it to be most suitable (**19**). Plates were exposed in a Pady-Rittis slit sampler (**11**), and a General Electric electrostatic spore sampler (**9, 10**) on the roof of one of the campus buildings 150 feet above ground. All exposures were made between 8:30 and 9:30 AM. A minimum of two plates was exposed daily in each of the two samplers. The manuals of Thom and Raper (**20**) and Raper and Thom (**17**) have been followed in determining species of both genera. A detailed description of the methods and materials as well as general results is given in a previous paper (**7**).

RESULTS AND DISCUSSION

The two genera, *Penicillium* and *Aspergillus,* constituted important components of the fungus population of the air. They ranked fourth

[1] Contribution No. 545, Department of Botany and Plant Pathology, Kansas Agricultural Experiment Station, Manhattan, Department serial No. 699. Part of a study of airborne fungi supported by the Kansas Agricultural Experiment Station and by grants (1380, 1380-C) from the U. S. Public Health Service.

[2] Respectively: assistant professor of botany; head, botany department, Kansas State University; and curator, Cryptogamic Herbarium, New York Botanical Garden. The assistance of graduate students R. Bhatti and V. K. Pathak, and the technical assistance and cooperation of Laurel Ouye, Joyce Murphey, Dorothy Bell, Bonnie Wiley, and Jeanette Arnett are gratefully acknowledged.

and fifth, respectively, in number of colonies obtained behind *Cladosporium* (44.5%), *Alternaria* (12.6%) and yeasts (8.4%). *Penicillium* with 6,950 colonies comprised 6.1% of the total colonies, *Aspergillus* with 6,146 colonies, 5.4%.

Daily average number of colonies of *Penicillium* per cubic foot of air is presented in FIG. 1. *Penicillium* follows a definite seasonal trend despite a great day-to-day variation. The highest numbers of colonies were obtained from July to November or December, with lower numbers occurring in late winter and early spring.

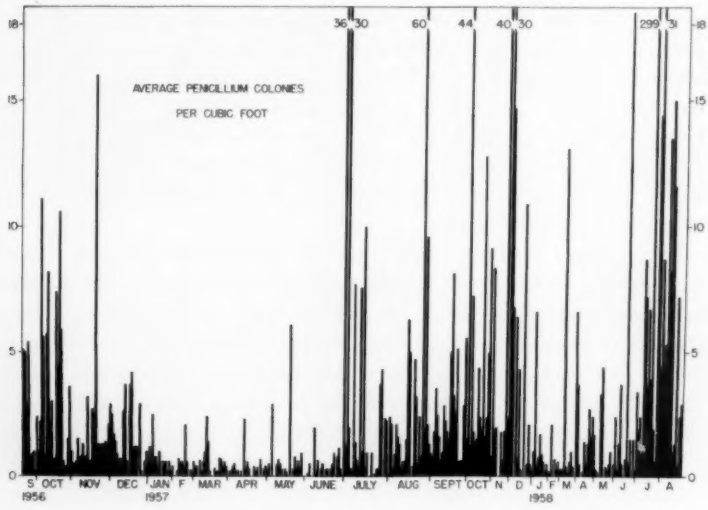

FIG. 1. Average number of colonies of *Penicillium* per cubic foot for each of the 403 sampling days from September, 1956, to September, 1958.

The extremes in daily variation of *Penicillium* are often caused by rainfall. Following a rain, the spore population of this genus is markedly decreased due to the rain removing the spores from the air. However, moisture causes a pronounced increase in sporulation which results in a rather sudden influx in the numbers of spores in the air during the following day or two. July 26, 1958, 0.86 in. of rain was received, followed by 1.0 in. on July 27, resulting in low colony counts (1.9 and 0.6/cu ft) the two following days. The third day, colony counts began to increase, reaching 14.4 colonies/cu ft July 30, and 31.0/cu ft July 31. In the afternoon of July 31 (after the exposures were made for that day),

0.92 in. of rain was received, resulting in a sudden drop in numbers of colonies (4.3/cu ft) the next day.

Aspergillus also follows a seasonal trend in occurrence (FIG. 2), with high numbers in spring and fall and low numbers in the summer. This becomes clearer when a comparison is made between monthly averages of *Penicillium* and *Aspergillus* colonies (FIG. 3), which indicates that *Penicillium* prefers warmer weather, whereas *Aspergillus* appears to be somewhat more abundant during cooler months. But these results are only from samples taken between 8:30 and 9:30 AM.

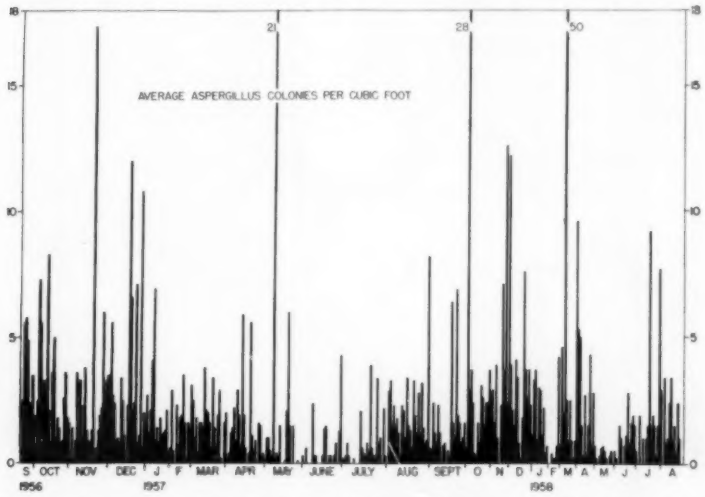

FIG. 2. Average number of colonies of *Aspergillus* per cubic foot for each of the 403 sampling days from September, 1956, to September, 1958.

Once a week, all colonies of a single RBS plate were subcultured and identified to species—referred to here as the "species plate" studies. From these plates a total of 35 species of *Penicillium* and 23 of *Aspergillus* was collected. The species and the number of times each occurred are listed in TABLE I.

Penicillium oxalicum Currie & Thom was by far the commonest species of this genus, representing 56% of the colonies collected from the "species plates." No other species formed more than 6% of the total colonies of this genus.

There were two species of *Aspergillus* that were exceedingly abundant, *A. niger* van Tieghem which formed 32% of the total colonies, and *A. amstelodami* (Mang.) Church & Thom which formed 23%. Too

little information is available about the occurrence of species of these fungi in the air for generalizing, but there seems to be some difference in the seasonal frequency of the two species. *A. amstelodami* appears to be somewhat more abundant during the growing season, whereas *A. niger* occurs more or less at the same frequency throughout the year. The occurrence of species of fungi in the air is in definite need of more study.

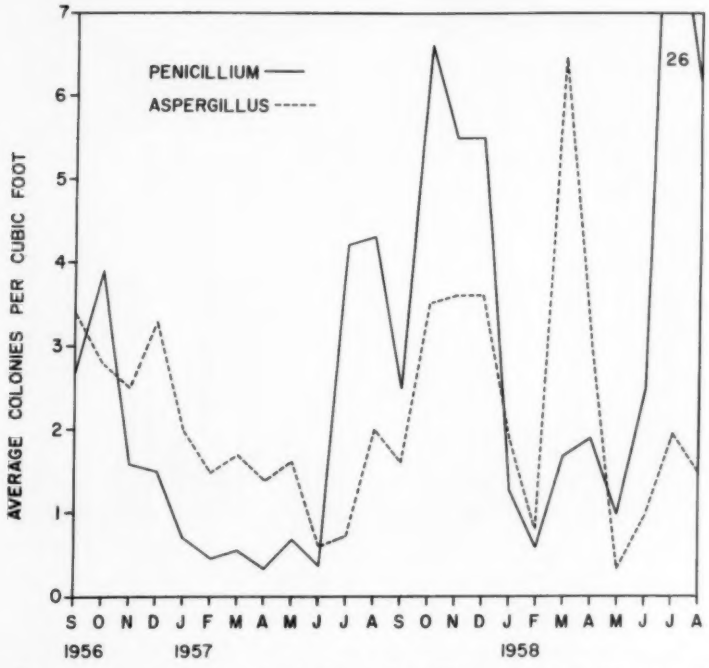

FIG. 3. Average number of colonies of *Penicillium* and *Aspergillus* per cubic foot of air for each month of the sampling period from September, 1956, to September, 1958.

Although the weekly "species plate" studies were the only systematic study of species, occasionally a particular colony appearing on other plates aroused a special interest and was identified. TABLE II is a list of such species.

A number of the species collected of these two genera were ascigerous forms. The perfect stage was produced in culture in 4 species of *Penicillium* and 7 of *Aspergillus*. These species are designated with asterisks in TABLES I and II.

TABLE I

SPECIES OF PENICILLIUM AND ASPERGILLUS OBTAINED FROM "SPECIES PLATE" STUDIES

Total colonies of *Penicillium*	271**	Total colonies of *Aspergillus*	222**
Penicillium canescens Sopp.	4	*Aspergillus alliaceus* Thom & Church	1
Penicillium chrysogenum Thom	16	**Aspergillus amstelodami* (Mang.) Thom & Church	51
Penicillium citreo-viride Biourge	1	*Aspergillus caespitosus* Raper & Thom	3
Penicillium commune Thom	1	*Aspergillus candidus* Link	1
Penicillium corylophilum Dierckx	2	*Aspergillus carneus* (v. Tiegh.) Bloch., emend.	1
Penicillium cyaneum (Bain. & Sart.) Biourge	1	**Aspergillus chevalieri* (Mang.) Thom & Church	6
Penicillium cyclopium Westling	8	*Aspergillus clavatus* Desm.	12
Penicillium decumbens Thom	2	*Aspergillus flavus* Link	16
Penicillium diversum Raper & Fennell	2	*Aspergillus fumigatus* Fres.	8
Penicillium duclauxi Delacroix	1	*Aspergillus mangini* (Mang.) Thom & Raper	2
Penicillium frequentans Westling	6	*Aspergillus melleus* Yukawa	2
Penicillium gladioli Machacek	1	**Aspergillus nidulans* (Eidam) Wint.	2
Penicillium herquei Bain. & Sart.	2	*Aspergillus niger* van Tieghem	72
Penicillium janthinellum Biourge	7	**Aspergillus quadrilineatus* Thom & Raper	1
Penicillium jenseni Zaleski	1	**Aspergillus repens* (Cda.) DeBary	5
Penicillium kapuscinskii Zaleski	2	*Aspergillus restrictus* G. Smith	5
Penicillium lanosum Westling	2	**Aspergillus ruber* Bremer	10
**Penicillium luteum* Zukal	1	**Aspergillus rugulosus* Thom & Raper	2
Penicillium multicolor Grigorieva-Manoilova & Poradielova	1	*Aspergillus sclerotiorum* Huber	2
Penicillium nigricans (Bain.) Thom	1	*Aspergillus sydowi* (Bain. & Sart.) Thom & Church	4
Penicillium notatum Westling	7	*Aspergillus terreus* Thom	6
Penicillium oxalicum Currie & Thom	152	*Aspergillus ustus* (Bain.) Thom & Church	4
Penicillium paxilli Bain.	1	*Aspergillus versicolor* (Vuillemin) Tiraboschi	6
Penicillium piscarium Westling	4		
Penicillium purpurogenum Stoll	1		
Penicillium pusillum Smith	1		
Penicillium rubrum Stoll	15		
Penicillium rugulosum Thom	2		
Penicillium simplicissimum (Oudemans) Thom	1		
Penicillium steckii Zaleski	4		
Penicillium stoloniferum Thom	1		
Penicillium tardum Thom	9		
Penicillium verruculosum Peyronel	4		
Penicillium waksmani Zaleski	2		
**Penicillium wortmanni* Klöcker	5		

** Number of times each species was identified from the "species plates."
* Species which developed the perfect stage in culture.

Several workers have reported that these two genera of fungi form one of the main components of air spora. Hyde and Williams (3) found that *Penicillium* formed 13% of the colonies obtained on gravity-exposed plates at Cardiff, Wales, being exceeded only by *Cladosporium*.

TABLE II

ADDITIONAL SPECIES OF PENICILLIUM AND ASPERGILLUS IDENTIFIED FROM PLATES OTHER THAN THE "SPECIES PLATES"

Penicillium casei Staub
Penicillium claviforme Bain.
Penicillium funiculosum Thom
**Penicillium levitum* Raper & Fennell
Penicillium lilacinum Thom
**Penicillium parvum* Raper & Fennell
Penicillium piceum Raper & Fennell
Penicillium restrictum Gilman & Abbott
Penicillium spinulosum Thom
Penicillium variabile Sopp

Aspergillus flavipes (Bain. & Sart.) Thom & Church
Aspergillus niveus Bloch., emend. Thom & Raper
Aspergillus ochraceus Wilhelm
Aspergillus sulphureus (Fres.) Thom & Church

* Species which developed the perfect stage in culture.

Aspergillus was considerably lower at 1%. *Penicillium* formed 4.9% of the colonies obtained by Richards (**18**) on gravity-exposed plates from several locations in Britain. Only *Cladosporium* (69.6%) and *Pullularia* (5.5%) were higher while *Aspergillus* was again considerably lower, forming only 0.9% of the colonies.

Pady and Kapica (**14**), when sampling air with the General Electric bacterial air sampler and the Bourdillon slit sampler at Montreal, Canada, found *Penicillium* and *Aspergillus* to form 15.8% and 10.4%, respectively, of the total colonies. These genera were exceeded only by *Cladosporium* (47.7%).

Wolf (**21**) in a series of exposures from airplanes over Nashville in 1943, obtained 2 cultures of *Penicillium*. The species were not identified but belonged to the group Asymmetrica. Four collections of *Aspergillus* were made belonging to 2 species, *A. terreus* Thom and to an unidentified species in the *A. niger* group near *A. luckuensis*.

Proctor and Parker (**16**) obtained 3 species of *Penicillium: P. frequentans* Westling, *P. glabrum* (Wehmer) Westling, *P. lanosum* Westling, and 6 species of *Aspergillus: A. fumigatus* Fries, *A. niger* van Tieghem, *A. flavus* Link, *A. glaucus* Link, and *A. calyptratus* Oudemans from the air at altitudes of 1500 to 16,500 feet.

SUMMARY

The genera, *Penicillium* and *Aspergillus,* formed two of the more important components of the fungus population of the air at Manhattan, Kansas, during a two-year study from September, 1956, through August, 1958. Results are based only on studies of colonies obtained by exposing plates of rose bengal-streptomycin agar in the Pady-Rittis and General Electric samplers since spores of these genera could not be identified from exposed silicone slides. *Penicillium* formed 6.1% of the total colonies while *Aspergillus* represented 5.4%.

The occurrence of both genera was greatly affected by climatic conditions. In general, however, *Penicillium* appeared to prefer warmer weather while *Aspergillus* preferred cooler temperatures.

A total of 41 species of *Penicillium* was identified, of which *Penicillium oxalicum* was the commonest, representing 56% of all colonies. *Aspergillus* was represented by 23 species, with *A. amstelodami* and *A. niger* formed 32% and 23%, respectively, of the total colonies.

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SOME SOIL AND COPROPHILOUS FUNGI FROM THE SOUTH PACIFIC AREA [1]

RONALD H. PETERSEN

Since Waksman's first exploratory paper on soil fungi and Actinomycetes (9), many reports have been written on the soil fungi from various parts of the world. Few have dealt with the south Pacific area, and, to the best of the writer's knowledge, none have described the soil fungi of Tahiti and Moorea. This paper reports on soil and dung fungi found on these islands.

MATERIALS AND METHODS

The soil and dung samples were collected in 1956 by Dr. L. S. Olive of Columbia University. Upon collection, they were placed in containers and air dried. After being returned to the United States, they were refrigerated until such time as they were plated out. Due to this extended period of storage, many fungi may have been lost.

Although only a few samples were used for this experiment, several techniques were employed. These were as follows:

No. 10059: Small quantities of pulverized soil were spread directly on plates of potato-dextrose agar.

No. 10061: 1. Serial dilutions up to 10^{-5} were pipetted in one cc quantities onto corn meal-dextrose agar. 2. Serial dilutions in peptone-glucose agar were blended for two seconds in a sterile Waring blender, then plated. 3. Direct inoculation of soil on both media was made. 4. Some soil was soaked in 0.05% NaOH solution for two hours, after which serial dilutions were plated on corn meal-dextrose agar.

No. 10147: Direct inoculation of soil was made onto plates of potato-dextrose agar, rose bengal-streptomycin agar, and V-8 juice agar.

No. 10222: Serial dilutions up to 10^{-3} in saline solution were plated on potato-dextrose agar and on dextrose-peptone agar.

No. 10224: Soil was inoculated onto plates of potato-dextrose agar, rose bengal-streptomycin agar, and Czapek's agar. It was soon apparent that *Penicillium corylophilum* was present in such quantity as to suppress or overgrow every other organism.

[1] Part of a thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts, Columbia University, New York City.

Nos. 10230, 10240: Soil suspensions were made in warm melted agar and plated on dextrose-sorbose medium, corn meal agar, potato-dextrose agar, and Czapek's agar.

No. 10234: 1. Serial dilutions in distilled water were made up to 10^{-5} and plated on potato-dextrose agar and Czapek's agar. 2. Warcup's technique (**10**) of pouring agar over soil or soil suspensions was used.

No. 10245: 1. Serial dilutions in 0.5% saline solution were plated on carrot-sucrose agar. 2. Serial dilutions up to 10^{-5} in sterile distilled water were plated on corn meal agar. 3. A duplication of the final technique used in number 10061 was used.

No. 10247: Serial dilutions up to 10^{-7} in sterile distilled water were plated on rose bengal-streptomycin agar, and V-8 juice agar.

With the dung samples, two techniques were employed. Dung suspensions were made in sterile distilled water and in 0.7% sodium acetate

TABLE I

SOIL AND DUNG SAMPLES FROM TAHITI AND MOOREA

1. Horse dung: Paea District, Tahiti	4/24/56
2. Horse dung: Afareaitu District, Moorea	4/18/56
8. Horse dung: Near Tautira, Tahiti	6/28/56
9. Cow dung: Punaauia District, Tahiti	7/56
10. Cow dung: Punaauia District, Tahiti	7/56
10059. Soil: Paea District, Tahiti; coffee plantation	4/24/56
10061. Soil: Pirae District, Tahiti; trail to cascades	4/8/56
10147. Soil: Hitiaa District, Tahiti	4/15/56
10222. Soil: Moorea; near Taro patch	3/29/56
10224. Soil: Moorea; under Mape tree	5/19/56
10230. Soil: Pirae, Tahiti; under Mape tree	4/8/56
10234. Soil: Arue District, Tahiti; soil around crab hole	4/15/56
10240. Soil: Paea District, Tahiti; moist river bed	4/24/56
10245. Soil: Paea District, Tahiti; coffee plantation	4/24/56
10247. Soil: Moorea; beneath Pandanus	3/29/56

solution. The suspensions were warmed under 60 watt bulbs for two hours, at which time serial dilutions up to 10^{-5} were made. These were plated on asparagine-dextrose agar, corn meal agar with 0.2% yeast extract, and Czapek's agar.

All colonies, when visible to the naked eye, were transferred to Czapek's agar. Subsequently, when necessary, fungi were cultured on other agars for identification. These agars were malt extract agar, bean agar, corn steep agar, wort agar and plain agar with cotton threads.

A list of soil and dung samples, with their origin and date of collection, is in TABLE I.

RESULTS

In this survey 69 species representing 25 genera were recovered. These species and their occurrence are given in the list of isolated fungi

TABLE II

LIST OF FUNGI ISOLATED FROM TAHITI AND MOOREA

Fungi	Substrata & Localities*
PHYCOMYCETES	
Cunninghamella bainieri Nau.	S/T
Rhizopus nigricans Ehrenb.	S/M, T
ASCOMYCETES	
Chaetomium bostrychodes Zopf	S/T
Chaetomium funicola Cooke	S/T
Chaetomium quadrangulatum Corda	S/T
Melanospora zamiae Corda	S/T
Sordaria fimicola (Roberge) Ces. & DeNot.	S, H/T
Sporormia minima Auersw.	H/T, M
Xylaria sp.	S/T
BASIDIOMYCETES	
Coprinus sp.	H/T
FUNGI IMPERFECTI	
Phomales	
Diplodia theobromae (Pat.) Nowell	S/T
Diplodia sp.	S/T
Moniliales	
Alternaria tenuis auct.	S/T
Alternaria sp.	H/T
Aspergillus clavatus Desm.	H/T
Aspergillus flavus Link	S/M
Aspergillus fonsecaeus Thom & Raper	S/T
Aspergillus fumigatus Fres.	S/T
Aspergillus niger van Tieghem	S, H, C/T, M
Aspergillus panamensis Raper & Thom	S/T
Aspergillus sydowi (Bain. & Sart.) Thom & Church	S/T
Aspergillus ustus (Bain.) Thom & Church	S/T, M
Aspergillus versicolor (Vuill.) Tiraboschi	S/T
Cephalosporium sp.	S/M
Cladosporium spp. I, II	S/T
Fusarium coeruleum (Lib.) Sacc.	S/T
Fusarium scirpi Lamb. & Fautr. var. *longipes* (Wr. & Rg.) Wr.	S/T
Fusarium spp. I, II, III	S/T
Geotrichum candidum Link f. *phytogena* Link	S/T
Geotrichum sp.	S/T, M
Gliocladium deliquescens Sopp	S/T
Graphium sp.	H/T
Hyalopus sp.	S/T
Mastigosporium heterosporum Petersen	S/T
Myrothecium striatosporium Preston	S/M
Paecilomyces varioti Bain	S/T
Penicillium atramentosum Thom	S/T
Penicillium camemberti Thom	S/T
Penicillium chrysogenum Thom	S, H/T, M
Penicillium corylophilum Dierckx	S, H, C/T, M
Penicillium expansum Link	S/T
Penicillium funiculosum Thom	S/T
Penicillium lanoso-coeruleum Thom	S/T
Penicillium lilacinum Thom	S/T
Penicillium miczynskii Zaleski	S/T
Penicillium notatum Westling	S/T
Penicillium oxalicum Currie & Thom	S/T
Penicillium resticulosum Birk., Rais. & Smith	S/M
Penicillium sublateritium Biourge	S/T
Penicillium variabile Sopp	S/T
Penicillium verruculosum Peyronel	S/T
Penicillium spp. I, II, III, IV	S/T, M
Stachybotrys subsimplex Cooke	S/T
Trichoderma koningi Oudemans	S/M
Trichoderma lignorum (Tode) Harz	S/T, M
Trichoderma sp.	S/T
Mycelia Sterilia	
Species I, II, III, IV, V	S/T, M

* Substrata: S = soil, H = horse dung, C = cow dung. Localities: T = Tahiti, M = Moorea.

(TABLE II). No data were recorded on the relative abundance of any one species, but merely the occurrence of each organism in each soil or dung sample.

In general, the best agar for identification was found to be Czapek's agar, only because species of *Penicillium* and *Aspergillus* and other genera have been described on this medium. The growth rate of most fungi on this medium was considerably less than could be desired. Potato-dextrose agar, malt extract agar, asparagine-dextrose agar and V-8 juice agar all supported rapid growth, but were often not suitable for identification purposes. Wort agar, corn steep agar, rose bengal-streptomycin agar, bean extract agar and plain agar with cotton threads, while frequently supporting an easily identified organism, permitted too slow a growth rate to be worthwhile.

Most of the fungi isolated were those expected to occur in this climate and section of the world. Others, however, are worthy of note because of the lack of reports on their occurrence in these substrata or geographical areas.

Geotrichum candidum f. *phytogena,* according to Saccardo (**7**), is native of Europe, but many workers consider it to be worldwide, but variable in oidial size.

Chaetomium bostrychodes and *C. funicolum* are not reported by either Palliser (**4**) or Greathouse and Ames (**3**) as occurring outside the United States. *C. quandrangulatum,* according to Chivers (**1**), also occurs only within the USA.

In the identification of *Chaetomium bostrychodes,* two articles were consulted, with a third being used as an auxiliary authority. The former articles were those of Palliser (**4**) and Greathouse and Ames (**3**), the latter by Chivers (**1**). The illustration given by Greathouse and Ames clearly shows the oral hairs of the perithecium to be branched as well as coiled. In the original description of *C. bostrychodes* and the descriptions given by all cited authorities, no mention is made of branching oral hairs. In fact, according to Miss Palliser, the broadest diagnostic feature within the genus is the presence or absence of branching oral hairs, *C. bostrychodes* being placed with the latter group.

Penicillium camemberti was not reported from soil by Gilman (**2**) or Raper and Thom (**6**), but my isolate appears to be that species.

Thom and Raper (**8**) draw special attention to *Aspergillus fonsecaeus,* both because of its large spores, and its apparently limited occurrence in South America. They report conidia measurements to be 5.5–8.5 μ in diameter; the conidia of the present collection are 6.2–9.2 μ in diameter.

Aspergillus panamensis is also native of South and Central America. Occurrence of this species, as well as *A. fonsecaeus,* in the south Pacific area is to be expected in view of the prevailing winds, currents and shipping between the two areas.

The recovery of *Myrothecium striatosporium* is worthy of note since it has been reported only once before, that time from New Zealand.

A phragmosporous fungus representing a new species of *Mastigosporium* was described earlier (**5**).

SUMMARY

Soil and dung from Tahiti and Moorea yielded 67 species, representing 25 genera. Some noteworthy species include *Aspergillus fonsecaeus, A. panamensis, Chaetomium bostrychodes, C. funicola, C. quadrangulatum,* and *Myrothecium striatosporium.*

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PLASMOGAMY AND ASCOCARP DEVELOPMENT IN GELASINOSPORA CALOSPORA[1]

JOHN J. ELLIS[2]

(WITH 23 FIGURES)

Sun (1953) was able to observe the development of perithecial initials of the homothallic strain of *Gelasinospora calospora* (Mouton) Moreau & Moreau in living cultures. She seemingly observed plasmogamy but was not able to follow development between the formation of a pseudoparenchymatous ball of cells and crozier formation. Most of the reported descriptions of the Pyrenomycetes state that development proceeds in the usual manner between these latter two stages. Meyer (1957) attempted to fill this gap in our knowledge of *G. calospora;* however, he made observations from only stained, sectioned material.

The purpose of the present investigation was twofold: firstly, to follow and record plasmogamy as it occurs in the homothallic strain of *G. calospora;* and secondly, to discover the origins of the various tissues and structures found in the protoperithecium and to follow their development.

Plasmogamy as used in this presentation refers to the fusion of protoplasts that provides the means by which compatible nuclei can come together prior to fusion.

There seems to be an inconsistency in the way various investigators have used the words coils, archicarp, protoperithecium, and ascogonium, and it is difficult at times to determine which structures are being discussed. In the present report, the word archicarp is not used. Protoperithecial initial refers to a simple structure consisting of several tight coils of an unbranched, septate or nonseptate hypha that ultimately gives rise to the various tissues of the perithecium. The word protoperithecium refers to the ball of hyphae or cells after the initial has branched forming inner and outer coils but before the asci have formed. Ascogonium refers to the inner coil which gives rise to the fertile layer.

[1] Portion of a thesis submitted to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Doctor of Philosophy, under the direction of Professor C. J. Alexopoulos.

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It seems less confusing to keep its original meaning as suggested by Kihlman (**1883**), that is, the "organ" that gives rise to the ascogenous hyphae and asci. *G. calospora* forms no extensive ascogenous hyphae; the croziers are formed from a compact tissue at the base of the immature perithecium. Andrus (**1936**) in his description of *Ceratostomella multiannulata* Hedgc. & Davidson thought that the terminal portion of the inner coil became the ascogonium; however, current usage of the term ascogonium seems to refer to the entire inner coil.

MATERIALS AND METHODS

A culture of *Gelasinospora calospora* designated as B-3 was obtained from Dr. C. J. Alexopoulos. This culture was originally obtained from Dr. Claude Moreau. A single-spore isolate from the B-3 culture designated as E-4 was used throughout this investigation. Stock cultures were maintained on agar slants in the refrigerator at 6° C.

All Petri dish cultures were grown at room temperature at approximately 25°. Cultures grown on microscope slides were incubated for three days at 20°. The slide cultures were supplied with a constant source of approximately 100 ft-c from a 15 w fluorescent bulb. On the fourth day they were taken out to room temperature for observation.

Difco corn meal agar supplemented with 0.5 g of Difco Bacto-peptone per liter was used throughout the investigations. On this medium, the fungus produces sparse aerial hyphae and scarcely any hyphal pigment, but fruiting is abundant. Abundant production of aerial hyphae or much pigment would interfere with observations of the initial stages of protoperithecial development.

The use of the Shoemaker slide (Shoemaker, **1950**) was adopted for observing the initial stages of protoperithecial development in culture. The inoculated slides were placed in Petri dish moist chambers and incubated in the temperature control cabinet. Later the slides were removed and placed on the stage of the microscope at 2, 4, or 6 hour intervals for observation. With this method, many coils formed adjacent to the cover slip and within 3 mm of the inoculated surface of the agar. They were readily seen and easily photographed after each of the first four successive intervals; however, further development ceased.

Temporary mounts to observe protoperithecia were made with the use of a large dissecting needle which was bent near the end and flattened to form a blade. Strips of agar were cut parallel to the surface of the culture approximately 2 mm thick and 1 cm wide. The strip of agar containing protoperithecia was placed, culture side up, on a micro-

scope slide, fixed and stained. In temporary mounts, the nuclei in the densely cytoplasmic protoperithecia could be observed only with the use of Giemsa stain following rapid fixation in osmium tetroxide vapors. Giemsa stain gave sharpest differentiation when used in the following schedule: Fix over 1% osmium tetroxide vapors for 30–45 seconds. Add a few drops of Kodak Photo-Flo diluted 1:50 in distilled water for 1½ minutes and rinse off with distilled water; Photo-Flo seems to minimize precipitation of the stain. Cover the mount with 2 drops of buffered, distilled water at pH 6.8 and add one drop of undiluted Giemsa stain. Allow 10–15 seconds for the stain to penetrate and wash off the excess stain with distilled water. Add 2 drops of 4% KOH for clearing and differentiating. In 5–10 seconds the color of the mount changes from a deep purple to blue. Wash the mount with distilled water, add a No. 1 cover slip, and smear. This procedure is carried out entirely on the microscope slide. The temporary mount can be observed immediately and usually lasts for several weeks if the cover slip is ringed with clear fingernail polish and the slide is kept in the refrigerator.

Temporary mounts for observing the initiation of croziers and paraphyses were made by using the propiono-carmine smear technique as described by Sass (**1951**). The slides were ringed with clear fingernail polish and allowed to age at room temperature for 24 hours before they were observed.

Material for permanent mounts was prepared by two methods. The first was to inoculate a thin layer (ca. 3 mm) of corn meal agar which covered one side of a microscope slide. These slides were incubated in Petri dish moist chambers until the time of killing and fixing. This method produced abundant protoperithecia but the mature perithecia were somewhat smaller and fewer than those produced on media in Petri dishes. The second method was to inoculate Petri dishes containing 20 ml of corn meal agar and incubate them at room temperature.

Agar coated slides and Petri dishes with media were inoculated at 24-hour intervals on 10 successive days. Also, on the fifth and sixth days additional slides and Petri dishes were inoculated to give 12-hour intervals. On the twelfth day, agar blocks 1 cm × 0.5 cm were cut, placed on a microscope slide and inverted over 1% osmium tetroxide vapors for 2 minutes. These blocks were then placed in small glass vials of formalin-propiono-alcohol killing and preserving fluid (Sass, **1951**) for 24 hours. They were dehydrated in the tertiary butyl alcohol series and imbedded in paraffin. Sections 8–10 μ were cut both parallel and perpendicular to the surface of the culture, giving cross and longitudinal sections of the developing perithecia.

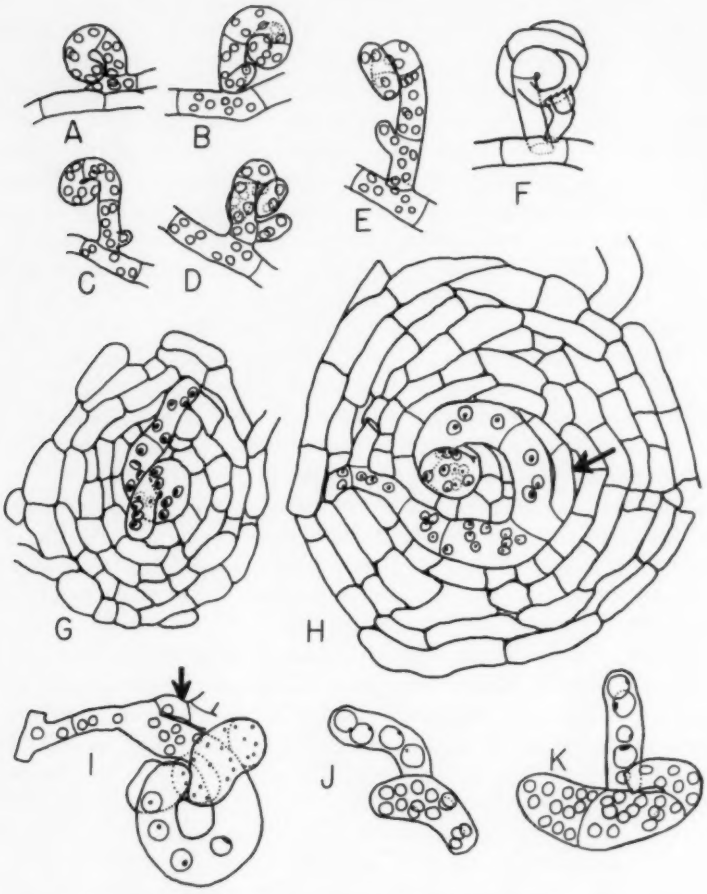


FIG. 1. *Gelasinospora calospora*. A. Protoperithecial initial showing the nuclear condition, × 500. B–E. Protoperithecial initials showing the first branching, × 500. F. Part of a protoperithecium showing the first branch and fusion with the mother cell, × 500. G. Section of a protoperithecium showing nuclear condition of the inner coil, × 750. H. Section of protoperithecium (FIG. 19) showing the first and second (arrow) branches of the inner coil, × 750. I. Drawing of protoperithecial section of FIG. 11 showing branching (arrow) and enlarged nuclei in the terminal portion of the ascogonium, × 750. J–K. Two primary ascogenous hyphae containing enlarged nuclei and successive stages of the branch containing numerous darkly staining nuclei, × 750.

Best nuclear and cell wall differentiation of the perithecia was obtained by using a modification of the 4 hr schedule iron-alum hematoxylin technique combined with fast green in clove oil as a counterstain. The technique is essentially that outlined by Sass (**1951**), with the following exceptions. The slides were left in the mordant and hematoxylin 5 hours each and were left overnight in distilled water before destaining.

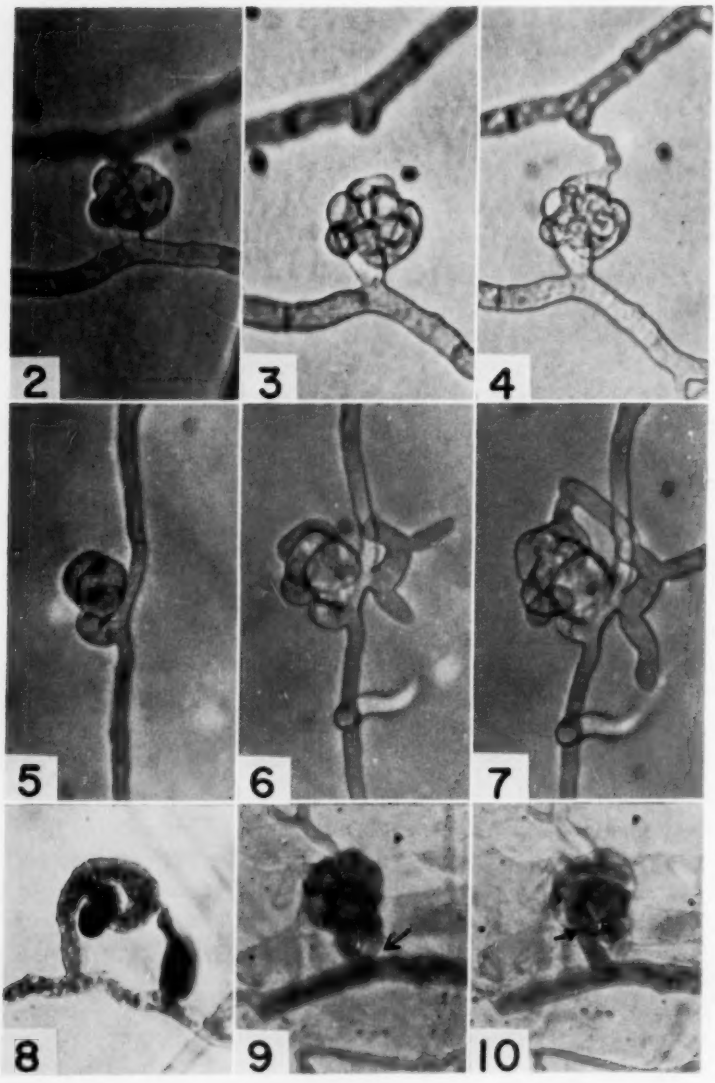
OBSERVATIONS

The Protoperithecial Initial.—The protoperithecial initials appeared as coiled lateral branches of the assimilative hyphae (FIG. 1 A–E). Under the cultural conditions used, protoperithecial coils formed approximately 80–90 hours after the corn meal agar was inoculated with hyphal tips. They formed first near the edge of the Petri dishes but soon could be found throughout the cultures. Plasmogamy took place within the next 6–12 hours, and by the time the protoperithecium was 24 hours old, it was an opaque ball of pseudoparenchymatous cells.

The protoperithecial coil arises usually as a short side branch from an intercalary cell of a hypha densely filled with cytoplasm. The tightly coiled protoperithecial initial becomes 1–3-septate, with each cell containing 3–9 nuclei (FIG. 1 A). A branch arises usually from the basal cell (FIG. 1 B–E) and forms the outer coil. Cells of the outer coil have a smaller diameter (ca. 3–5 μ) than those of the inner coil (ca. 4.5–6 μ). The origin of the protoperithecium from the mother hypha and the first branch forming the outer coil can be seen in FIG. 10 and are represented in FIG. 1 F. The nuclear situation at this stage could not be determined.

Plasmogamy.—Plasmogamy occurs shortly before or, more frequently, shortly after the formation of the first branch. It was found to occur in any of three ways. First, a short peg may form on a nearby portion of an adjacent hypha and a filament from the terminal portion of the inner coil may grow out and fuse with the peg (FIGS. 2–4). A second way in which plasmogamy occurs is by fusion of a filament from the mother hypha with a short peg of the inner coil (FIGS. 5–7, 8). The third way is by fusion of a hyphal branch from the terminal portion of the inner coil with the mother hyphal cell (FIGS. 1 F, 9).

The Protoperithecium.—Soon after plasmogamy has taken place, subsequent development of the ascogonium in living material is obscured by the enveloping layer of cells of the outer coil. The inner coil, or ascogonium, can be seen in the pseudoparenchymatous ball when it is stained with propiono-carmine or Giemsa stain and smeared. The as-



FIGS. 2–10. *Gelasinospora calospora.* FIGS. 2–4. Growth of a protoperithecial initial during which a filament from the coil fused with a peg on a nearby hypha. During the interval between FIG. 2 and FIG. 3, the agar on the microculture slide had split separating the two hyphae. FIGS. 5–7. Growth of a second protoperithecial initial during which a hypha from the mother cell fused with the terminal portion

cogonium appears as a densely stained, coiled hypha of larger diameter than the cells surrounding it. Sections of paraffin-imbedded material stained with iron-hematoxylin also showed this differentiation (FIG. 19). The fact that the ascogonium stains more densely than the surrounding cells indicates that the inner coil remains as a differentiated structure.

The outer coil, which arose as a branch from a basal cell of the protoperithecial initial, proliferates to a compact ball of pseudoparenchymatous cells surrounding the inner coil. These cells contain 2–7 nuclei each. The outer coil eventually becomes the outer wall of the mature perithecium. Numerous long tapering hyphae grow out from the outer layer of the pseudoparenchymatous cells, extend in all directions, and fuse with other hyphae growing in the area near the developing protoperithecium. It is assumed that these hyphae provide the means by which the outer tissues of the protoperithecium obtain nutrient necessary for growth and development.

The ascogonium becomes septate (FIG. 1 G, H), each cell remaining multinucleate throughout the development of the protoperithecium. The terminal portion of the ascogonium enlarges slightly in diameter and elongates 6–8 fold; it is densely cytoplasmic, and its cells contain 3–9 nuclei. At this stage some of the nuclei could be interpreted either as being paired or as having just divided. All nuclei were not paired; on the other hand, division figures were not found.

As development proceeds, a second branch arises from the inner coil (FIGS. 1 H, I, 11, 19) and surrounds the multinucleate terminal cells of the ascogonium (FIGS. 13–15). This branch consists of many short cells containing 2–6 nuclei each. The hyphae arising from the second branch ultimately become the inner wall tissue of the mature perithecium. The terminal cells of the inner coil give rise to the primary ascogenous hypha and the tissue that forms the paraphyses.

The Ascogenous Hyphae.—Two, usually four, or sometimes six nuclei approximately 5 μ in diameter can later be found in the terminal cell of the ascogonium (FIG. 1 I). They are approximately twice the diameter of darkly staining nuclei found in the adjacent cell of the ascogonium and have smaller nucleoli than the darker staining nuclei.

The terminal cell of the ascogonium is interpreted as the primary ascogenous hypha. This cell is encircled by the hyphae from the second

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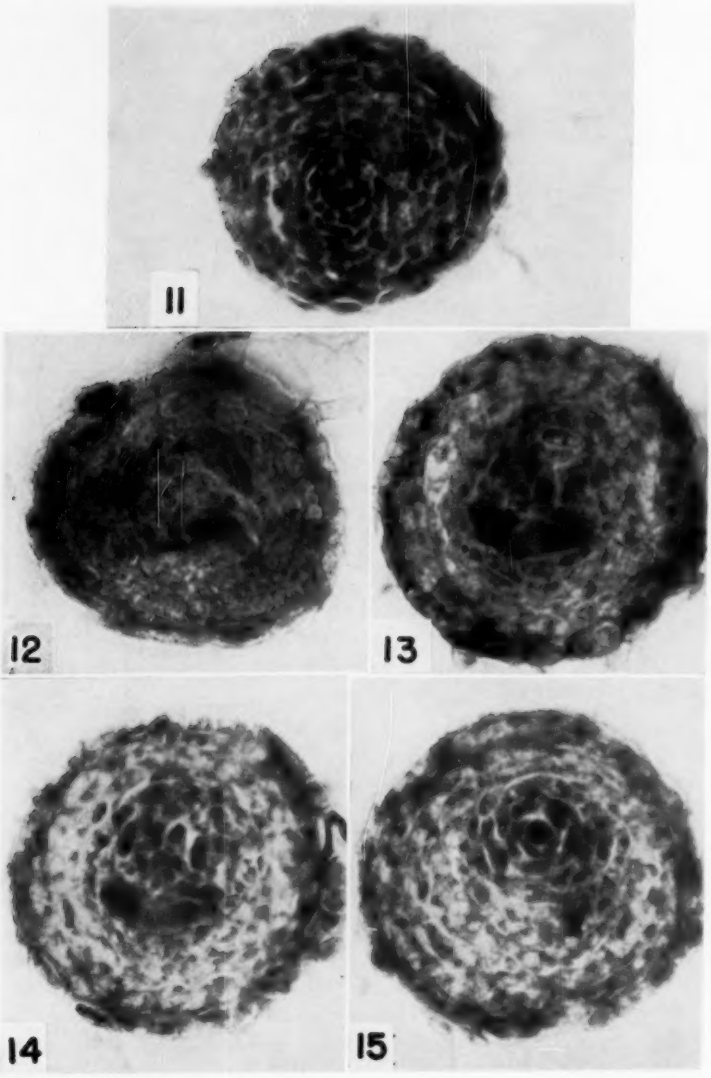
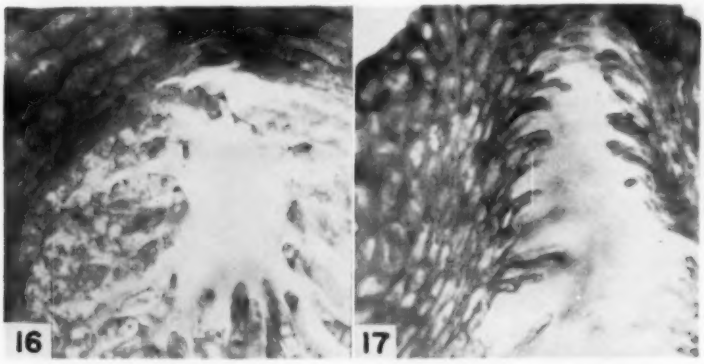
of the inner coil. FIG. 8. Hyphal branch fusing with a peg on a protoperithecial initial. FIGS. 9, 10. Two optical sections of the same protoperithecium shown in part in FIG. 1 F. Arrow in FIG. 9 shows fusion. Arrow in FIG. 10 shows origin of first branch. All figs. × 500.



FIGS. 11–15. *Gelasinospora calospora.* FIG. 11. One of the serial sections of a protoperithecium from which composite drawing of FIG. 1 I was taken, × 500. FIG. 12. One of the serial sections of a protoperithecium from which composite drawing of FIG. 1 J was taken, × 500. FIGS. 13–15. Three successive sections of the same protoperithecium from which composite drawing of FIG. 1 K was taken, × 500. FIG. 13 is bottom-most; FIG. 15 is top-most.

branch of the ascogonium (FIG. 15). The encircling hyphae become further septate and pseudoparenchymatous (FIG. 20). It is in this area of the developing protoperithecium that the cavity begins to form (FIG. 20). At this time, the primary ascogenous hypha gives rise to a mass of cells, and very short hyphae from these cells give rise to asci by typical crozier formation.

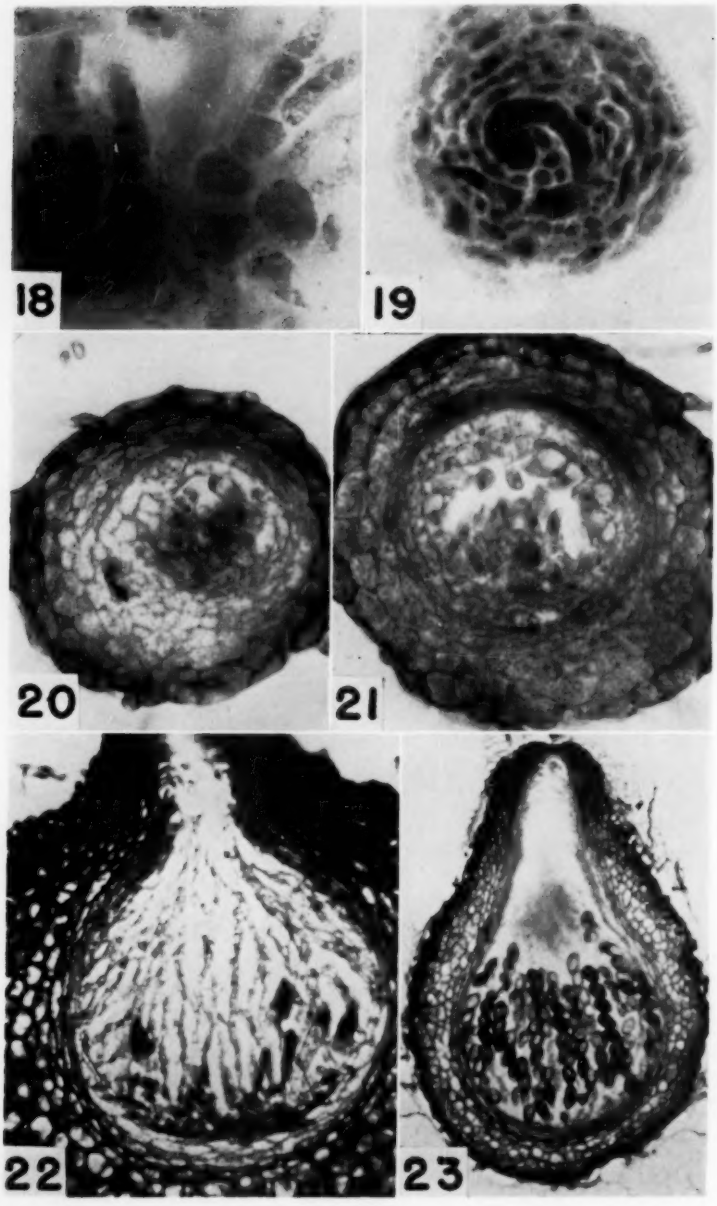
Formation of Paraphyses.—As many as 24 darkly staining nuclei may be present in the subterminal cell of the ascogonium or a branch thereof. While the primary ascogenous hypha is still present, this nearby cell enlarges and becomes septate (FIGS. 1 J, K). The precise origin of the enlarged cell containing the darkly staining nuclei could not be determined.



FIGS. 16, 17. *Gelasinospora calospora.* FIG. 16. Longitudinal section of young perithecium showing paraphyses proliferating from cells along inner wall and compressed area at the top where periphyses initiate from the inner wall, × 650. FIG. 17. Later stage of perithecial neck showing young periphyses as extensions of inner wall cells, × 650.

Immediately before the cavity begins to form and the first croziers can be found, paraphyses begin to proliferate. They arise from the multinucleate cells formed by the septation of the penultimate cell of the ascogonium. One or two paraphyses originate from each cell (FIG. 18). The paraphyses are septate and grow parallel to one another; each of their cells contains 2–5 nuclei. The perithecium at this time is spherical and there is no internal or external evidence of ostiole formation (FIG. 21).

After further development, the tissue that gives rise to the paraphyses lies between a cluster of young asci and the base of the perithecium.



FIGS. 18–23.

The asci are surrounded basally by the young paraphyses, and by using the smear technique, it is easy to separate the cluster of asci from the surrounding tissues. The paraphyses form higher and higher along the inner perithecial wall (FIG. 16). Before the asci mature, the paraphyses nearly fill the cavity; and by the time the asci contain ascospores, the paraphyses have become an almost unrecognizable mass by dissolution.

Formation of the Cavity.—At the time the first croziers and paraphyses appear, the cavity begins to form by the separation of hyphal elements in the region immediately above the fertile layer (FIG. 20). The size of the perithecium increases by an increase in number of cells and tangential expansion of the outer cells. Soon an inner ring of tissue two to three cells thick can be seen in a longitudinal section of the perithecium (FIG. 21). This hollow sphere of cells forms the inner wall of the perithecium. The cavity expands dorsally as the ostiole forms.

Ostiole Formation and Periphyses.—Narrow hyphae round off from the flattened, densely cytoplasmic cells of the inner wall at the top of the perithecium (FIG. 16) and become the first periphyses. The ostiole extends outward as the neck of the perithecium is formed by the upward growth of the periphyses and the outer wall cells (FIG. 17). When the perithecium nears maturity, the cavity opens to the outside and the neck appears as an inverted cone. By this time, the first formed periphyses as well as the paraphyses appear to have undergone some dissolution (FIG. 22).

The Mature Perithecium.—In a longitudinal section of a mature perithecium (FIG. 23), three well-defined layers are present. The outermost layer is 1–2 cells thick; its cells have heavily pigmented walls at least on the outer side. The next layer is 3–5 cells thick and consists of relatively thin-walled cells. These two layers comprise the outer wall of the perithecium. The inner wall layer is 1–3 cells thick and lines the entire cavity; its cells have thin walls and appear tangentially elongated. The 8-spored asci nearly fill the perithecial cavity.

The perithecia matured first near the periphery of the Petri dish

FIGS. 18–23. *Gelasinospora calospora.* FIG. 18. A smear showing the origin of paraphyses, × 1000. FIG. 19. Photograph of protoperithecial section shown in drawing of FIG. 1 H, × 500. FIG. 20. Section of protoperithecium showing initiation of cavity, × 500. FIG. 21. Section of protoperithecium showing ring of inner wall tissue. Paraphyses are approximately the same stage as shown in FIG. 18, × 500. FIG. 22. Section of immature perithecium showing beginning of dissolution of paraphyses, × 250. FIG. 23. Mature perithecium, × 75.

colony; they did not all develop simultaneously. Some initials remained as coils and failed to develop further; their cells became filled with vacuoles. Some protoperithecia did not mature but formed small sterile spheres of pseudoparenchymatous cells. These spheres attained approximately one-fifth the diameter of mature perithecia. Their outer cells were thick-walled and heavily pigmented, and the inside was entirely filled with thin-walled cells.

DISCUSSION

The time of the appearance of protoperithecial initials in *G. calospora* seems to differ little from that observed for *Neurospora tetrasperma* by Colson (**1934**). She reported the "simultaneous" appearance of the initials in her cultures; in the present investigation, the initials formed within a period of six hours. Very few formed as late as six hours after the first initials appeared.

There seems to be no morphological difference between the basal cells and the terminal cells of the protoperithecial initial. Each cell is multinucleate as was reported for *Neurospora tetrasperma* by Colson (**1934**) and Gilles (**1950**), and for *Sordaria fimicola* by Greis (**1936**). Both Gilles and Greis reported nuclear division in the initial, but applications of their methods in the present investigation showed no convincing mitotic figures in *G. calospora*. Interphase nuclei appeared well defined with the smear techniques.

It is certain that plasmogamy occurs in *G. calospora*. In several instances living material on microculture slides showed the lone coil first and, several hours later, the coil after fusion had taken place. Successive stages were photographed in two of those instances; numerous other instances of plasmogamy were seen in smear preparations. Meyer (**1957**) observed an anastomosis between a protoperithecial initial and another hypha and found another in which a branch of a "pseudotrichogyne" had fused with the mother hypha. He did not believe that this was plasmogamy, however, since he did not find these anastomoses frequently.

The situation in *G. calospora* was found to be similar to that reported for *Gelasinospora reticulospora* (Greis) Moreau (syn. *Rosellinia reticulospora* Greis) by Greis and Greis-Dengler (**1940**). These authors reported two types of plasmogamy: anastomosis between an antheridium and ascogonium, and anastomosis between a pseudotrichogyne and an assimilative hypha. They also reported apparent nuclear autogamy in the ascogonial cells. Four trichogyne-like structures were found in *G.*

calospora during the present study. In one instance, the filament arose from the tip of a coil; in another it arose subterminally on the coil; in a third instance, the tip of the trichogyne was fused with a neighboring hypha; and, two short branches of a neighboring hypha were fused with a trichogyne in the fourth instance. Thus, plasmogamy may infrequently occur by this means in *G. calospora*.

The first branch from the protoperithecial initial was previously seen by Meyer (**1957**) and has also been reported in related genera (*Sordaria* by Greis **1936**, *Neurospora* by Gäumann **1952**, and *Melanospora* by Cookson **1928**). Cookson (**1928**) stated that the inner wall of *Melanospora zamiae* forms from cells of the inner coil immediately above those concerned with the production of the outer coil. This corresponds with the observations made on *G. calospora* in the present study.

Formation of the perithecial wall from parts of the initial coil, or from hyphae involved in the protoperithecial initial, has been reported previously in *G. calospora* by Meyer (**1957**) and in several other Pyrenomycetes (Colson **1934**, Greis **1936**, McGahen and Wheeler **1951**, and Zickler **1952**). However, there has previously been no mention of the origin of the various tissues from successive branches of the inner coil.

Meyer (**1957**) did not report the origins of the inner wall tissue and paraphyses from successive branches of the inner coil, nor did he show the persistent basal portion of the original protoperithecial initial. He believed that the original "ascogone" persisted, but that the pedestal became drawn out and degenerated. If this is the case, the basal portion degenerates late in the development of the protoperithecium and near the time that the cavity begins to form. FIGURES 1 I, 11 show the latest stage at which the basal portion of the inner coil was seen in the present study.

The finding of different size nuclei in the ascogonium confirms the report of Meyer (**1957**); however, the enlarged nuclei were found only in the terminal portion of the ascogonium in the present investigation. Meyer believed that the enlarged nuclei were in prophase of mitosis, although the details of division figures were difficult to interpret. No observations in the present study support his findings.

The significance of the larger nuclei in the ascogonium is not certain, but sectioned material indicates that the cells containing these nuclei divide to become the fertile layer. The enlarged nuclei or derivatives thereof are the functional nuclei which eventually divide conjugately in the croziers. They were seen not only in the ascogonium of *G. calospora* but also several times in some of the cells of the fertile layer at the base of the protoperithecium shortly before formation of croziers and paraphy-

ses. The croziers seemed to begin their formation from a few cells in this compact layer. Conjugate division was seen in the croziers.

Andrus and Harter (**1933**) found no structures resembling ascogenous hyphae in *Ceratostomella fimbriata,* but Andrus (**1936**) later found cells in *Ceratostomella* similar to those mentioned above in the compact layer of *G. calospora*; he doubted that such a cell could properly be called an ascogenous hypha. Blackman and Wellsford (**1912**), Gilles (**1950**), Brooks (**1910**), and Jones (**1926**) have found enlarged nuclei in the inner coils of other Pyrenomycetes.

Sterile spheres similar to those found in the present study have also been reported for *Neurospora* (Shear and Dodge **1927**, Colson **1934**, and Dodge **1935**) and *Sordaria* (Greis **1936**). Colson (**1934**) described these spheres as "abortive" protoperithecia. No evidence of an ascogonium was found in sections of these spheres in the present investigation, supporting Colson's belief.

Meyer (**1957**) reported that as the perithecium of *G. calospora* develops, the paraphyses arise from cells above the ascogonium. Observations in the present study indicate that they arise from cells in the base of the protoperithecium below the fertile layer.

There seems to be good evidence that *G. calospora* develops a well defined perithecium. The perithecia are produced as separate structures on the assimilative hyphae, all structures of the perithecium arise from the initial coil, the paraphyses represent newly produced hyphae which are free at their tips, and the periphyses represent newly produced hyphae formed from the inner wall.

Direct evidence supporting the independent origin of the various perithecial tissues was obtained by observation of their branching from the ascogonium. The fact that the various tissues could be easily separated from one another at various stages of perithecial development by using dissecting needles and smear techniques may be cited as indirect evidence. The outer wall of the perithecium could be easily separated from the inner wall, the asci from the inner wall and from the paraphyses, and the paraphyses from the inner wall. The periphyses could not be easily separated from the inner wall, indicating that they are an extension of the inner wall tissue.

The perithecial development of the homothallic strain of *Gelasinospora calospora* most closely resembles that group described by Luttrell (**1951**) as having the *Xylaria*-type centrum in which the compact fertile tissue lies in the base of the perithecial cavity, the asci arise as an aparaphysate cluster, and the evanescent paraphyses line the perithecial wall.

SUMMARY

The structures of the homothallic strain of *Gelasinospora calospora* involved in plasmogamy and perithecial development are similar to those found in species of *Sordaria* and *Neurospora*, especially *N. tetrasperma*. *G. calospora* has a centrum which resembles that described by Luttrell as one kind of the *Xylaria*-type centrum.

The origins of the various perithecial tissues were found; their development was followed in stained sections and smear preparations. The ease with which certain of these tissues were separated suggested their independent development.

The protoperithecial initial arises as a tightly coiled side branch from an intercalary hyphal cell. The initial becomes 1–3-septate; each cell contains 3 to 9 nuclei. A branch arises usually from the basal cell of the protoperithecial initial, envelops the initial, and becomes the outer coil of the protoperithecium. Plasmogamy takes place usually after the branch initiates and most frequently occurs by the fusion of a short branch of the mother hypha or a neighboring hypha with a short peg formed on the inner coil.

The outer coil proliferates into a compact layer of multinucleate pseudoparenchymatous cells which surrounds the inner coil. Numerous hyphae grow out from the outer cells of the ball and make protoplasmic connections with the surrounding hyphae. The inner coil (ascogonium) enlarges in diameter, elongates, and becomes less tightly coiled. Septa form in the ascogonium leaving 3 to 9 nuclei in each cell.

A second branch arises from the ascogonium and becomes a network of thinner hyphae which surrounds the terminal cells of the ascogonium. The short multinucleate cells of these hyphae become compact and tangentially elongated forming the inner wall of the young perithecium.

Two to 6 nuclei in the terminal portion of the ascogonium enlarge to approximately twice their original diameter. This portion of the ascogonium becomes the primary ascogenous hypha. Enlarged nuclei were also seen in the ascogenous tissue at the base of the young perithecium and seemed to be the nuclei which eventually paired in the croziers. Cells immediately above the primary ascogenous hypha separate and thus initiate the perithecial cavity.

A third branch from the ascogonium, containing up to 24 darkly staining nuclei, enlarges and becomes septate. The resulting cells produce the paraphyses which proliferate higher and higher along the inner wall.

Cells of the inner wall round off and initiate the periphyses at the

top of the perithecium. The periphyses and outer wall tissue form the neck of the perithecium. Ostiole formation proceeds from the cavity to the outside.

At maturity, the perithecium consists of an outer wall, an inner wall which extends as periphyses into the neck region, gelatinized remnants of the paraphyses, and asci containing eight pitted ascospores each.

ACKNOWLEDGMENT

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CONDITIONS AFFECTING GROWTH AND INDIGOTIN PRODUCTION BY STRAIN 130 OF SCHIZOPHYLLUM COMMUNE [1]

NORMAN S. SWACK AND PHILIP G. MILES

(WITH 1 FIGURE)

The production of a blue, water insoluble pigment by certain mutant cultures of the basidiomycete *Schizophyllum commune* Fries was first observed by Papazian (**4**) in association with a mutant character termed *streak*. Although pigment production in the fungi is a fairly common occurrence and such phenomena are frequently of taxonomic importance, the production of a blue pigment is relatively rare. In *S. commune* the mutation from the normal, nonpigmented condition to forms producing pigment is evidently common. Some 46 blue pigment producing strains from 13 stocks collected in widespread geographical locations are known. The blue pigment has been identified by Miles, Lund, and Raper (**3**) as indigotin.

Many interesting phenomena have been observed in connection with this pigment. The mating of certain pigment producing strains (e.g., *early blue*) with a nonpigment producing strain gives progeny having a one to one ratio of pigmented to nonpigmented isolates, whereas other pigment producing strains (e.g., *late blue*-1 and *late blue*-2) when mated with a normal strain produce from thirty to forty per cent indigotin producing isolates (**5**).

Indigotin is also produced by certain bacteria. Gray (**1**) has demonstrated that various soil bacteria (*Pseudomonas indoloxidans, Mycobacterium globerulum, Micrococcus piltonensis*) are able to oxidize indole to indigotin when grown in a medium containing indole. Indigotin production by *S. commune* does not require the addition of indole to the medium, however.

[1] This investigation was supported in part by a research grant (E-1518) from the National Institute of Allergy and Infectious Diseases, Public Health Service.

Most of the work reported here formed part of a thesis submitted by the senior author to the Graduate School of Arts and Sciences of the University of Buffalo in partial fulfillment of the requirements for the degree of Master of Arts, April, 1958.

Although much is known about the artificial synthesis of indigotin, very little is known concerning the biosynthesis of this compound; therefore, the production of this unusual compound by *Schizophyllum commune* affords an opportunity for such studies. *S. commune* is an excellent organism to use for the elucidation of the biochemical pathway of indigotin synthesis because of the ease with which the organism can be handled in culture, the fact that it can be grown and will produce pigment on a chemically defined medium, and the existence of a certain amount of background in the application of the techniques of biochemical genetics to this organism.

It would be of value to determine the nutritional conditions which affect the synthesis of this pigment. The goals of these investigations are the discovery of the optimal conditions for growth and development of pigment and the attainment of some insight into the pathway by which this synthesis occurs.

MATERIALS AND METHODS

Studies were made using a single pigment producing strain of *Schizophyllum commune* (no. 130), a het-mutant from a stock collected at Big Sur, California, by Professor John R. Raper.

The organism was grown in 250 ml Erlenmeyer flasks containing 50 ml of a minimal liquid medium of the following composition:

Glucose	10	g
$(NH_4)_2HPO_4$	1.5	g
Thiamine	120	μg
KH_2PO_4	0.46	g
K_2HPO_4	1.0	g
$MgSO_4 \cdot 7H_2O$	0.5	g
Distilled water	1.0	liter

Studies involving different carbon sources were made by substituting an equivalent amount of the carbon source to be studied for the glucose present in the minimal medium. In order to prevent caramelization of the carbon sources, each was sterilized separately and added aseptically to sterile medium containing no other carbon source for growth.

The utilization of different nitrogen containing compounds was studied by substituting for the ammonium phosphate these various nitrogen compounds in amounts which gave an equivalent nitrogen content to that in the minimal medium.

Inoculations were made by macerating a 3.5 cm diam culture, grown on minimal agar medium, in 50 ml of sterile distilled water in a Waring blendor. One ml of this macerate was then transferred aseptically to

each flask. Cultures were grown on a reciprocating shaking machine at a temperature of 27° C for two or three weeks, or at 30° C for three weeks. The cultures were then studied to determine the pH of the medium, the dry weight of the mycelium, and amount of indigotin produced. Normally one-half of the cultures were used for dry weight determinations and the other half were used for the analysis of pigment. To determine the dry weight of the mycelium the entire culture was filtered on tared filter paper, washed three times with distilled water, dried and weighed.

The indigotin produced was prepared for analysis by adding five ml of concentrated HCl to each culture and heating these cultures to 121° C in the autoclave for ten minutes. This treatment was necessary to break down a polysaccharide which forms during growth (**6**). Early in this study it was found that the pigment adhered to the polysaccharide during the analysis thus preventing complete extraction of the indigotin. After it had cooled, the acid-treated culture was filtered through Schleicher and Schuell No. 589 Red Ribbon filter paper. The pigment and mycelium collected in this manner were then washed three times with distilled water and allowed to dry. The pigment was extracted with hot acetone in a continuous Soxhlet-type extractor until no trace of blue remained on the filter paper. When the extraction appeared to be complete, the flask was heated until all the acetone was driven off. This treatment left a residue in the extraction flask containing the pigment. This residue was treated with concentrated sulfuric acid in preparation for colorimetric analysis according to a method devised by Lotichius and Kooyman (**2**). Small amounts of sulfuric acid were also added to the culture flasks since it was observed that a portion of the pigment usually adhered to them. This treatment converted the indigotin to the water soluble disulfonate of indigotin.

The contents of the culture flask and of the extraction flask were then combined and diluted to 2 liters. A 10-ml aliquot of this solution was read at 605 mμ on a spectrophotometer. The reading obtained was compared with a standard reference curve to determine the amount of indigotin produced. This standard reference curve (FIG. 1) was obtained from a series of solutions containing known concentrations of disulfonate of indigotin.

To determine the per cent recovery and thus the expected accuracy of the procedure in the presence of mycelium, minimal medium containing known amounts of indigotin was inoculated with strain no. 699, a nonpigment producing form, and these cultures were grown on the shaking machine for two weeks. The results of the assays for pigment

demonstrated a recovery of more than 80 per cent of the pigment in analysis. Extractions were made of mycelium (strain no. 699) in the absence of pigment. In all determinations zero optical density readings were obtained at 605 mμ; the presence of mycelium thus does not interfere significantly with the readings obtained.

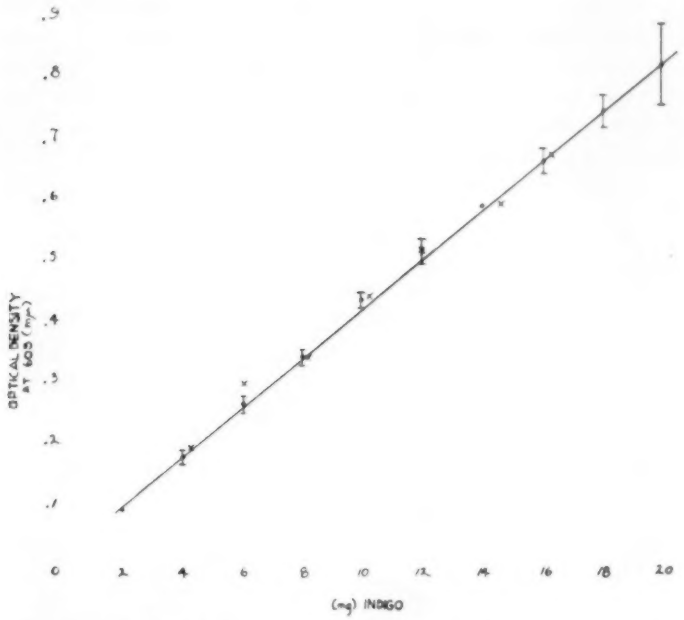

FIG. 1. Standard reference curve of disulfonate of indigotin. • represents an average of 3 optical density values (separate experiments) used to obtain the reference curve. Φ indicates the limits of 2× the standard error from the mean. If a normal distribution is assumed, 95.44% of chance deviations should fall within these limits. x represents optical density reading of known amount of indigotin.

All experimental cultures which did not produce pigment were subcultured to minimal agar plates and were checked microscopically after growth to confirm their ability to produce pigment.

RESULTS

Nitrogen Sources.—The utilization of inorganic nitrogen sources was studied by substituting KNO_3, $NaNO_2$, KCN, and NaSCN in amounts

equivalent in nitrogen for the $(NH_4)_2HPO_4$ in minimal medium. Cultures containing no nitrogen source other than the very small amount present in vitamin B_1 served as blank controls. Only the medium containing the ammonium ion as the nitrogen source produced mycelium and pigment.

Organic nitrogen sources were likewise substituted in amounts equivalent in nitrogen for the $(NH_4)_2HPO_4$ in minimal medium; these substitutions were based upon the assumption that each, with the exception of tryptophane, contained 16 per cent nitrogen. The results are summarized in TABLE I. Tryptophane was of interest in this study because it was thought that one of the precursors of indigotin might be indole and, hence, the indole + serine → tryptophane conversion found in other organisms (**8**) might be operative here. In the tryptophane cultures a

TABLE I

GROWTH AND PIGMENT PRODUCTION BY SCHIZOPHYLLUM COMMUNE NO. 130 IN MINIMAL MEDIUM CONTAINING EQUIVALENT AMOUNTS OF VARIOUS NITROGEN SOURCES. CULTURES GROWN ON SHAKER AT 27° C FOR THREE WEEKS

Nitrogen source	Average dry weight (mg)	Average indigotin produced (mg)
Peptone	42.0	1.7
Tryptone	75.5	22.0
Tryptophane	52.8	*
Tryptophane + $(NH_4)_2HPO_4$	52.7	*
$(NH_4)_2HPO_4$	27.0	14.4

Deviations for dry weight values were not more than 5.0 mg and for pigment values were not more than 0.5 mg.

* Acetone extraction produced a red color. Cultures grown in media containing tryptophane + $(NH_4)_2HPO_4$ also produced the red color with traces of indigotin present, but were not analyzed for pigment because of this red color.

red color appeared upon acetone extraction as was also true in the medium containing $(NH_4)_2HPO_4$ in addition to tryptophane. This strain of *S. commune* has been known to produce a red pigment before (**3**), but it was only encountered in minute amounts with larger quantities of indigotin. The values for dry weight and pigment were found to be greatest in the medium containing tryptone, which is rich in tryptophane. In the medium containing peptone, the small amount of indigotin produced in spite of abundant mycelial growth suggested that there might be something in peptone which inhibited pigment production. Dialyzed peptone was incorporated into agar medium upon which various indigotin producing mutants, including strain no. 130, were inoculated. Microscopic observation did not reveal greater pigmentation on the dialyzed than on the nondialyzed peptone medium. The explanation for the low production of indigotin on peptone medium remains unknown.

TABLE II

GROWTH, PIGMENT PRODUCTION AND TERMINAL pH OF THE MEDIUM PRODUCED BY SCHIZOPHYLLUM COMMUNE NO. 130 IN MINIMAL MEDIUM CONTAINING EQUIVALENT AMOUNTS OF VARIOUS FIVE-CARBON CARBOHYDRATES. CULTURES GROWN ON SHAKER AT 30° C FOR THREE WEEKS

Carbon source	pH	Average dry weight (mg)	Average indigotin produced (mg)
D-Arabinose	7.0	*	—
L-Arabinose	6.5	46.0	3.2
D-Ribose	6.5	48.5	4.2
D-Xylose	6.0	80.7	6.1
D-Glucose†	6.0	30.7	3.3

Deviations for dry weight values were less than 10 mg and for pigment values were less than 2 mg.

* Dry weight values less than 10 mg.

† Glucose determination for comparative purposes.

Carbon Sources.—Studies involving carbon sources are divided into three general groups, namely, those in which the glucose in minimal medium has been substituted by equivalent amounts of: 1) five-carbon carbohydrates, 2) six-carbon carbohydrates, and 3) carbohydrates of more than six carbon atoms. The results of these studies are summarized in TABLES II and III. Each of the groups contains a culture grown in glucose minimal medium for comparative purposes. The determinations were made in groups of three. Three cultures were analyzed for dry weight of the mycelium and three were analyzed for amounts of

TABLE III

GROWTH, PIGMENT PRODUCTION AND TERMINAL pH OF THE MEDIUM PRODUCED BY SCHIZOPHYLLUM COMMUNE NO. 130 IN MINIMAL MEDIUM CONTAINING EQUIVALENT AMOUNTS OF VARIOUS CARBOHYDRATES. CULTURES GROWN ON SHAKER FOR TWO WEEKS AT 27° C

Carbon source	pH	Average dry weight (mg)	Average indigotin produced (mg)
Cellobiose	5.4	70.1	6.0
D-Fructose	6.5	55.9	9.3
D-Galactose	6.3	58.9	6.8
D-Glucose	5.7	56.0	7.9
Lactose	6.8	*	—
Maltose	6.1	58.5	5.3
D-Mannitol	6.4	44.8	4.0
Raffinose	6.7	25.2	2.8
L-Rhamnose	7.0	*	—
D-Sorbitol	6.5	63.5	7.3
L-Sorbose	7.0	*	—
Sucrose	5.3	55.9	6.7

Deviations for dry weight values were less than 10 mg and for pigment values were less than 1.0 mg.

* Dry weight values less than 10 mg.

pigment produced. Values given in the tables are average values for three replicates.

Five-Carbon Carbohydrates. No pigment and no significant growth were present in the medium containing D-arabinose as the sole carbon source. Growth and pigment production did occur on L-arabinose, as well as the other five-carbon carbohydrates studied, with the highest values resulting when D-xylose was the carbon source. This study of five-carbon carbohydrates was the only one made at an incubation temperature of 30° C which was originally thought to be optimum. The cultures were allowed to incubate for three weeks. Other carbohydrates were studied at 27° C and cultures were analyzed after incubation for a two week period (TABLE II).

Six-Carbon Carbohydrates. No pigment or significant growth occurred in L-rhamnose or L-sorbose. Growth and pigment production occurred in all of the remaining six-carbon carbohydrates studied (D-galactose, D-fructose, D-mannitol, D-sorbitol, and D-glucose) (TABLE III).

Carbohydrates of More Than Six Carbon Atoms. All of the carbohydrates studied except lactose are able to serve as carbon sources for growth and pigment production. These included cellobiose, maltose, sucrose, and raffinose (TABLE III).

The Effect of Temperature.—Cultures grown for three weeks at 30° C with glucose as the carbon source produced approximately one-half the growth and pigment as that obtained from cultures which were grown for two weeks at 27° C. Thus high temperatures are apparently inhibitory for growth of this strain. Qualitatively analyzed studies showed that growth and indigotin production will occur at a temperature range of 2 to 37° C with best results obtained between 20 and 30° C.

The Effect of pH.—In all cases where pigment production occurred, the pH of the medium at the time of analysis was always less than 7. Previous studies in this laboratory have indicated that growth will not occur in an alkaline medium. Studies in which growth and pigment production were measured at different time intervals revealed that the pigment was produced in the pH range of from 6.3 to 4.0. During growth strain no. 130 always produced acid. The range of pH under which indigotin is produced is apparently wide.

DISCUSSION

This study demonstrates that strain no. 130 can be classified as a Group III organism according to the system of Robbins (7). Accord-

ingly, plants which fall under the Group III heading are capable of assimilating ammonia or organic nitrogen but are unable to use elemental nitrogen or nitrogen in the form of nitrates and probably nitrites.

The production of a red pigment by strain no. 130 was previously reported by Miles, Lund, and Raper (3). The red pigment was present along with indigotin, with the indigotin present in a greater amount. In the tryptophane medium the red pigment is relatively abundant. The production of indigotin by *Schizophyllum commune* differs from indigotin production by *Pseudomonas indoloxidans* as reported by Gray (1) in that the bacterium did not produce either indole or indigotin in a medium in which tryptophane was the energy source. If indigotin production by *S. commune* proceeds by way of indole, it is apparent that the indole comes from some other source than tryptophane breakdown although this may be responsible for some of the indole. It may be that the breakdown of tryptophane leads to a precursor for the red pigment. It is clear too that *S. commune* is less demanding in its nutrient requirements which will lead to indigotin production than is *P. indoloxidans*.

The different carbon sources studied demonstrate that the organism is able to utilize a variety of carbohydrates as the sole carbon source and that a carbohydrate which is utilized for growth is also suitable for the production of indigotin. Comparatively, it appears that D-xylose is the best carbohydrate of those studied for growth and is one of the best for pigment production, but glucose is superior or at least equal to the other carbohydrates investigated. During the course of this study it was observed that growth and pigment production in minimal medium which has been caramelized appeared earlier than minimal medium which is uncaramelized (sugar autoclaved separately). It is possible, therefore, that a breakdown product of glucose may be more suitable for growth and pigment production than is glucose itself.

The results obtained from the studies involving D-xylose incorporation in the medium are interesting from both a chemical and an ecological point of view. Structurally D-xylose is very similar to D-glucose. Xylose is found in hemicelluloses and in the xylans of woody tissue, and, since *S. commune* is found in nature as a wood-rotting fungus, it may be that a medium containing xylose more closely simulates conditions which the organism may encounter in its natural habitat.

Although these studies have not produced information which would of itself suggest a plausible biosynthetic pathway for the production of indigotin, some of the conditions required for growth and pigmentation and the nutrients which will favor these have been determined. The production of a red pigment which is favored by the presence of trypto-

phane may, with the positive identification of the pigment, be an important clue in determining the biosynthesis of indigoid pigments.

SUMMARY

A method for the quantitative determination of indigotin production by *Schizophyllum commune* which involves extraction with hot acetone is described. The amounts of pigment produced were determined colorimetrically by comparison with an indigotin standard reference curve.

Ammonia appeared to be the only inorganic nitrogen source utilized, but the organism was not limited with respect to organic nitrogen. Growth in a medium containing tryptophane resulted in the production of a red pigment.

Of the carbohydrates studied D-xylose gave the highest value for the production of mycelium, D-fructose and D-glucose gave the highest values for the production of pigment, and the sugars D-arabinose, L-rhamnose, L-sorbose, and lactose were not utilized.

Strain no. 130 of *Schizophyllum commune* grows best at temperatures below 30° C in an acid medium.

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PARASITISM OF GONATOBOTRYUM FUSCUM ON SPECIES OF CERATOCYSTIS [1, 2]

ALEX L. SHIGO [3]

(WITH 8 FIGURES)

INTRODUCTION

The recent investigations by Barnett and Lilly (**1958**), Butler (**1957**), and Berry (**1958**) on the physiology of mycoparasites have presented valuable information aiding in the elucidation of the basic principles of parasitism. Many additional interesting questions have arisen as a direct result of their investigations. Environment, host nutrition, and host species are important factors known to affect the degree of parasitism.

The fungi lend themselves very well to basic studies on parasitism, since they can be grown easily in a minimum of time and space, can be placed under many exacting conditions, and their response to differences in nutrition and environment can be measured accurately.

The genus *Gonatobotryum* was erected by Saccardo in 1877 for the species *G. fuscum* Sacc. The type collection was collected from decaying oak wood. Ainsworth et al. (**1945**) reported finding *G. fuscum* for the first time in England. It was found growing on oak wood that had been cut for fuel. Vincent (**1953**) reported *G. fuscum* as a parasite on *Ceratocystis* spp., growing under the loose bark of felled beech trees in England. No detailed information on this association was reported and no additional information has been found in the literature.

Hughes (**1953**) described *G. fuscum* as having pale brown conidiophores with terminal and intercalary swollen cells that bear conidia on short denticles. The conidia are pale brown and are borne in chains of two. Other morphologically related species, *Gonatorrhodiella highlei*

[1] Published with approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper No. 633.

[2] Condensed from a portion of a dissertation submitted to the faculty of the Graduate School of West Virginia University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

[3] Forest Pathologist, U. S. Department of Agriculture, Forest Service.

and *G. parasitica* have been reported as mycoparasites (Ayres, **1941**; Blyth, **1950**).

Although many examples of mycoparasites are reported in the literature, there are few accounts of the mode of parasitism, the effects of environment, and other important physiological factors that have a profound effect on the host-parasite complex.

The purpose of this paper is to report the results of investigations on *Gonatobotryum fuscum* as a parasite of other fungi and the conditions which modify its parasitic activity.

MATERIALS AND METHODS

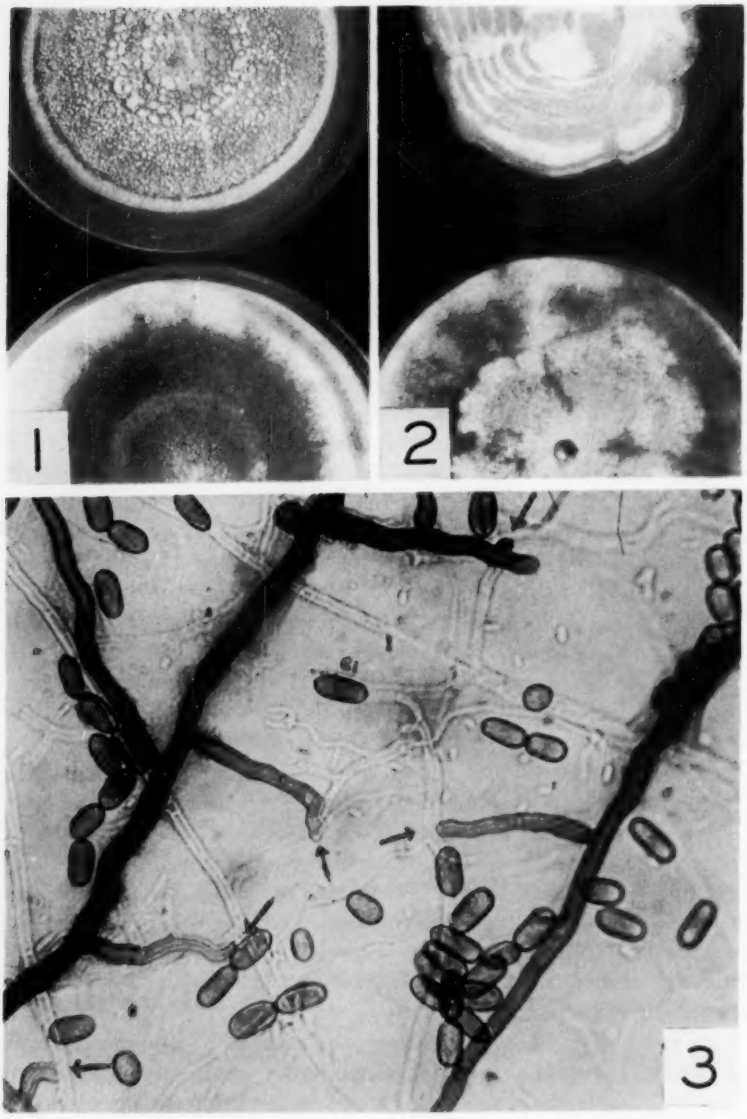
The isolate of *G. fuscum* was obtained by the author in November, 1956, while he was studying the fungi associated with trees killed by oak wilt in West Virginia (Shigo, **1958**). It was observed parasitizing a species of *Graphium* growing on an oak-wilt fungus mat cut from a red oak tree in Wetzel County, West Viriginia. The fungus was later obtained from thirteen trees in six other counties. The host species is uncertain and is referred to in this paper as *Graphium* sp. The identity of *G. fuscum* was verified by Dr. S. J. Hughes from a culture growing on the original host (FIGS. 2, 3).

Since axenic cultures could not be obtained, inoculum was maintained by growing the parasite on *Graphium* sp.

The majority of the fungi tested in the host range studies came from the stock-culture collection at West Virginia University. Several fungi were obtained from Baarn, Netherlands, and from other sources.

A wide variety of media were used and each will be discussed in connection with the experimental results. The medium that was found to be most suitable for the culturing of all fungi and for obtaining inoculum of the parasite contained 10 g malt extract, 2 g yeast extract, 20 g agar and one liter of distilled water, and is termed a 10–2 malt-yeast medium in this paper. A similar designation is also used to indicate the amounts of glucose and yeast extract in other media. In semi-synthetic media, 100 μg of pyridoxine per liter was added to assure normal growth and sporulation of *Graphium* sp.

In order to facilitate microscopic observations of the fungi growing in culture, cellophane disks, 90 mm in diam, were cut to fit inside a Petri dish. The disks were then boiled in water for twenty minutes to remove certain toxic materials and to sterilize them. Under aseptic conditions they were placed on agar in Petri dishes, and several ridges were formed so that the cellophane was not completely in contact with the



FIGS. 1–3. 1. *Graphium* sp. and *G. fuscum* (below) and host alone (above) after 20 days. 2. *Ceratocystis ulmi* and *G. fuscum* (below) and host alone (above) after 20 days. 3. *G. fuscum* (dark hyphae) parasitizing *C. ulmi* (hyaline hyphae), × 430. Points of contact are indicated by arrows.

agar. The inoculum was placed on the surface of the cellophane and as the fungi grew over these ridges, the mycelium became sparse. The cellophane ridge was cut into small pieces and placed on a microscope slide for observation. The cellophane method facilitated the observations of the hyphal connections between *G. fuscum* and its hosts.

Additional methods pertaining to specific experiments will be described later.

EXPERIMENTAL RESULTS

Establishment of G. fuscum as a Mycoparasite. *Gonatobotryum fuscum* has been observed in nature only in association with *Graphium* sp. and all early attempts to culture it free from a living host failed. The spores were placed on several different media and observed for as long as one month. A high percentage of the spores germinated, and a few germ tubes grew into hyphae several mm long, but growth soon ceased. Both of the spores in the two-spored chain germinated. Spores of *G. fuscum* mixed with those of *Graphium* sp. germinated quickly and the parasite mycelium could be seen within three days on a 10–2 malt-yeast medium at 25° C.

Microscopic examination revealed that there were connections between numerous small specialized branches of *G. fuscum* and hyphae of *Graphium* sp. (FIGS. 3, 4). These branches, called absorptive hyphae, were firmly attached to the host. No penetration of the host mycelium was observed. These absorptive hyphae were of several different shapes, the most common form being a holdfast-type (FIG. 4). The majority of the absorptive hyphae were formed on the young parasite hyphae and attacked the young host hyphae more readily.

A definite pattern of growth was always formed on the 10–2 malt-yeast agar medium when the host and parasite were inoculated at one spot. *Graphium* sp. began to grow quickly at 25° and produced an abundance of secondary spores. Synnema formation was stimulated by light, and under twelve hours alternating light and darkness, rings of synnemata were formed within a few days. During the first three to five days, the growth appeared as a pure culture of *Graphium* sp. The steel gray aerial mycelium of *G. fuscum* appeared at four to six days. The cultures soon took on a dark color that increased in intensity until they appeared almost black (FIG. 1). The parasite kept pace with the host and disrupted formation of the rings of dark synnemata. Only secondary spores were then produced by the host.

The parasite did not visibly inhibit the growth of the host. After several weeks the cultures took on a new macroscopic appearance. Abun-

dant aerial growth and luxuriant sporulation of *Graphium* sp. on the aerial hyphae and conidiophores of *G. fuscum* caused the cultures again to appear light colored. The tall conidiophores of *G. fuscum* then began to collapse. Microscopic examination showed the narrow hyphae of *Graphium* sp. twining around and penetrating the aerial hyphae of *G. fuscum* (FIGS. 6, 7). Many small dark spots were observed on the *G. fuscum* hyphae where *Graphium* sp. apparently attempted to penetrate. Scattered synnemata were often present throughout the cultures at this time.

This reversal of parasitism by *Graphium* sp. did not appear to be very destructive to *G. fuscum,* since only the aerial hyphae were affected.

Host Range of G. fuscum. Forty-five fungi were tested as possible hosts of *G. fuscum.* Spores of *G. fuscum* were collected by touching a bit of agar on a needle to a conidial head. They were placed on a 10–2 malt-yeast agar medium with spores or mycelium of the test fungi. Only fungi belonging to the genus *Ceratocystis* or related imperfect fungi were found to be hosts (TABLE I). To prove that these fungi were susceptible, they were all grown on cellophane and the presence of parasitic connections was verified.

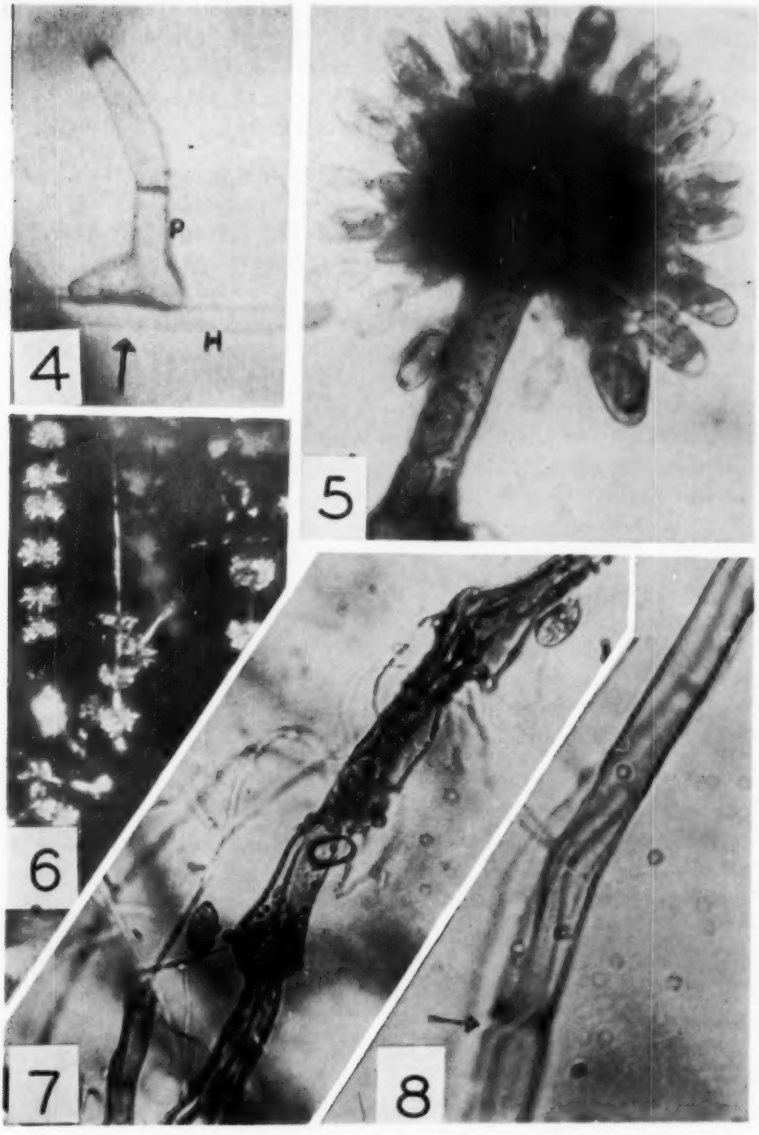
The degree of parasitism seemed to differ on the different hosts.

TABLE I

FUNGI TESTED AS POSSIBLE HOSTS FOR GONATOBOTRYUM FUSCUM

Ceratocystis fimbriata	+*	*Beauveria bassiana*	—
C. pluriannulata	+	*Penicillium frequentans*	—
C. pluriannulata variant	+	*Penicillium notatum*	—
C. fagacearum	+	*Aspergillus niger*	—
C. fagacearum albino mutant	+	*Oedocephalum* sp.	—
C. ips	+	*Gelasinospora* sp.	—
C. pilifera	+	*Stysanus media*	—
C. variospora	+	*Curvularia* sp.	—
C. virescens	+	*Phoma* sp.	—
C. ulmi	+	*Cephalosporium diospyri*	—
C. coerulescens	+	*Zygorhynchus moelleri*	—
Graphium sp.	+	*Fusarium oxysporum*	—
Graphium rigidum	+	*Dothiorella ulmi*	—
Leptographium sp.	+	*Dothiorella quercina*	—
Isaria cretacea	—	*Verticillium dahliae*	—
Chalaropsis sp.	—	*Mortierella pusilla*	—
Myrothecium roridum	—	*Gonatobotrys simplex*	—
Ascocybe grovesii	—	*Cladosporium* sp.	—
Stysanus stemonitis	—	*Alternaria tenuis*	—
Sporocybe sp.	—	*Physalospora ilicis*	—
Gliocladium roseum	—	*Petriella asymmetrica*	—
Trichothecium roseum	—	*Petriella* sp.	—

* Hosts are indicated by + and nonhosts by −.



FIGS. 4–8. *G. fuscum*. 4. Absorptive hypha (P), contacting host (H), × 970. 5. Sporulating node, × 970. 6. Conidiophores, × 60. 7. Conidiophore parasitized by *Graphium* sp., × 970. 8. Aerial hypha penetrated by *Graphium* sp., × 970.

Ceratocystis ulmi proved to be very useful in further studies since the parasite grew as far as twenty mm beyond the periphery of the host colony (FIG. 2). This was a desirable feature which made spores and hyphal tips easily obtainable without host contamination. The parasite markedly restricted the growth of *C. ulmi* and *Leptographium* sp.

Attempts at Axenic Culture of G. fuscum. Using spores and large pieces of agar containing mycelium and conidiophores of *G. fuscum,* many attempts were made to obtain an axenic culture of the parasite. The use of eighty-five different media resulted in general failure. The parasite grew to a diameter of 25 mm on several media, but the mycelium and conidiophores were sparse. It was evident that some nutrient essential to continued growth was lacking. Spores and mycelium from axenic cultures one month old were still capable of attacking hosts on fresh media.

Autoclaved and sterile-filtered mycelial extracts and culture filtrates from several hosts were added to mycelium of *G. fuscum,* but with limited success. The greatest amount of growth was obtained on media containing host-filtrates, small amounts of yeast extract, and microelements. The hyphae of *G. fuscum* frequently penetrated deeply into the agar, and thick, branching, aborted, nonsporulating conidophores were formed. Abnormal conidiophores on the agar surface produced oddly shaped spores, and often several branches originated from a single node. Under no condition did the parasite make continuous growth so that it could be transferred and form new mycelium in the absence of a living host.

Effects of Light and Temperature on G. fuscum. The effects of light and temperature on parasitism were determined using *C. ulmi, Leptographium* sp., and *Graphium* sp. as hosts. Each was inoculated with spores and mycelium of *G. fuscum* on a 10–2 malt-yeast agar medium. The cultures were incubated at 10, 15, 20, 25, 28, 30 and 35° C. The following light conditions were used: constant light, constant darkness, and 12 hours darkness alternating with white light, red light, and blue light. The data were obtained from at least six replicate cultures under each condition.

Growth of *G. fuscum* after twenty days at 10 and 15° was poor on *Leptographium* sp., fair on *C. ulmi,* and good on *Graphium* sp. These cultures were incubated in a refrigerator and received light only when the door was opened to examine the cultures.

The majority of the light experiments were conducted at 20°, since preliminary investigations indicated that this temperature was near

optimum for growth of the parasite. The average diameter of colonies of *Graphium* sp. after 8 days at 20° was 35 mm, and the cultures appeared approximately the same under all light conditions. The parasite grew far beyond the edge of the host only in total darkness. The effect of alternating blue light and darkness on growth of *G. fuscum* was similar to that of alternating white light and darkness. Few synnemata of *Graphium* sp. were produced under alternating red light and darkness and parasitism was fair to good under this condition. The mycelia of host and parasite grew deeply into the agar under continuous light, and parasitism was poor.

Parasitism of *Graphium* sp. was good in total darkness and poor under alternating light and darkness after eleven days at 28°. When several of the cultures grown under alternating light and darkness were placed in darkness for two days, the parasite grew rapidly beyond the host's periphery.

Only *C. ulmi* grew well at 30°. The parasite was absent under all conditions. After eleven days the cultures were placed in subdued light at 20°. Although these conditions stimulated good host growth, the parasite did not grow. But, when the original agar inoculum blocks from the cultures of hosts plus parasite incubated at 30° were placed on a fresh medium at 20°, normal host and parasite growth was noted in a few days.

Total darkness favored growth of the parasite on all hosts at 25°. While darkness stimulated parasitism, it suppressed synnema formation of *Graphium* sp. The presence of *G. fuscum* also disrupted synnema formation of *Graphium* sp. under the conditions otherwise favorable for their formation. These results indicate a close relationship between metabolites for growth of the parasite and synnema formation of *Graphium* sp.

The Effect of the Carbon-Nitrogen Ratio on the Degree of Parasitism. The ability of a parasite to infect a suitable host is dependent to a great extent on host nutrition (Brown, **1955**; Berry, **1958**; Barnett and Lilly, **1958**). The following experiments were designed to determine the effect of the carbon-nitrogen ratio on the degree of parasitism. The cultures were incubated at 25° in alternating 12 hours light and darkness. While preliminary experiments indicated that a malt extract-yeast extract medium was best for growth of this parasite on its hosts, glucose was also used as a carbon source.

The degrees of parasitism of *G. fuscum* on *Graphium* sp. on 24 media having different ratios of malt extract, and glucose, to yeast extract are

represented in TABLE II. The data were obtained from twelve colonies on each medium.

Host growth was approximately the same on malt extract and glucose media and was favored by relatively high concentrations of carbon. On the other hand, growth of the parasite was generally favored by relatively low concentrations of yeast extract. *G. fuscum* appeared in media containing as much as 5 g of yeast extract only when 20 g or more of malt extract was present. The greatest restrictions of host growth occurred in low concentrations of nutrients.

Leptographium sp. and *C. ulmi* were also used as hosts in some experiments. The parasite failed to develop on *C. ulmi* and *Graphium* sp.

TABLE II

GROWTH OF GONATOBOTRYUM FUSCUM AND GRAPHIUM SP. IN MEDIA HAVING DIFFERENT RATIOS OF PRINCIPAL INGREDIENTS

Ratio in grams per liter	Malt extract and yeast extract, 10 days		Glucose and yeast extract, 14 days	
	Parasite	Host alone	Parasite	Host alone
1–0	2*	1	3	1
0–1	2	1	3	1
1–1	2	2	4	1
1–5	0	2	0	3
1–10	0	1	0	2
5–1	4	3	2	3
5–5	0	3	0	3
10–1	4	3	0	3
10–2	4	4	3	4
20–1	4	3	2	4
20–5	4	3	0	3
40–10	1	3	0	3

* 0 = absent, 1 = poor, 2 = fair, 3 = good, 4 = excellent.

on a 5–5 glucose-yeast extract medium, but it grew well on *Leptographium* sp. on the same medium.

Gonatobotryum fuscum usually grew beyond the hyphal tips of *C. ulmi* on media containing malt extract, but did not do so when grown on the media containing glucose. Possibly some substances in malt extract, such as the microelements, may have been the cause of its superiority over glucose.

The carbon-nitrogen ratios as well as the specific hosts are important factors in the host-parasite complex. Different host-parasite combinations did not respond in the same way in media containing the same

amounts of carbon and nitrogen indicating that host physiology governs, to a certain extent, the degree of parasitism.

To expand the investigations on the effect of the carbon-nitrogen ratio on the degree of parasitism, several different nitrogen sources were used in amounts of nitrogen equivalent to the same weight of nitrogen in yeast extract. Glucose was the carbon source in all media. The 8 nitrogen sources are listed in TABLE III. For uniformity, the following were added to each liter of the 8 agar media: 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 ml of a microelement solution (0.2 mg each of iron and zinc, 0.1 mg of manganese and 20 mg of calcium) and 100 μg of pyridoxine.

The data on *G. fuscum* and *Graphium* sp. were recorded after ten days (TABLE III). All media supported good growth of *Graphium* sp.,

TABLE III

DEGREES OF PARASITISM OF GONATOBOTRYUM FUSCUM ON GRAPHIUM SP. IN AGAR MEDIA VARYING IN NITROGEN SOURCE AND IN CARBON-NITROGEN RATIO. CULTURES WERE INCUBATED 10 DAYS AT 25° C AND RECEIVED 12 HOURS LIGHT EACH DAY

g C source-N source	Nitrogen source							
	Yeast extract	Glutamic acid	Casamino acids	Urea	Potassium nitrate	Asparagine	Ammonium nitrate	Ammonium tartrate
5–5	4*	0	3	0	2	0	3	3
5–1	3	3	3	2	2	3	3	3
1–1	2	3	2	2	2	2	2	2
10–1	3	3	3	2	2	3	2	3

* 0 = absent, 1 = poor, 2 = fair, 3 = good, 4 = excellent.

and fair to good growth of the parasite, with the exception of the high concentration of glutamic acid, urea and asparagine.

Effects of Added Microelements on Parasitism. The vigorous growth of the parasite on hosts on the medium designated as 5–5 glucose-yeast extract was unexpected. In previous experiments the parasite had failed to grow on a similar medium without the added nutrients listed above (compare TABLES II, III). It was quickly realized that this medium had KH_2PO_4, $MgSO_4 \cdot 7H_2O$, microelements, and pyridoxine added to assure uniformity with the other media.

To verify the above results, *G. fuscum* and the two hosts, *Graphium* sp. and *C. ulmi,* were grown on a 5–5 glucose-yeast extract medium, with and without the additives mentioned above. The parasite was not evident on the two hosts on the medium without this supplement but

made good growth when the additional elements were present. By adding each nutrient separately to a 5–5 glucose-yeast extract medium, it was found that the microelements were the effective nutrients. The addition of 0.5 g per liter of $MgSO_4 \cdot 7H_2O$ to the medium also resulted in a small amount of parasite growth.

To determine whether one specific metal was necessary, the following metals were added separately, in amounts equal to that received in 2 ml of the microelement solution, to the 5–5 glucose-yeast extract medium: iron, zinc, manganese, and calcium. Data obtained from fifteen replicates of *G. fuscum* on *Graphium* sp. after twelve days indicated that manganese was the necessary metal. The addition of manganese alone was as effective as the combination of the microelements. The diameters of host growth were uniform on all media, but differences in sporulation of the host were evident. Zinc, copper and calcium suppressed synnema coloration and formation. Similar results were obtained using *C. ulmi* as the host, and the manganese-amended medium improved growth of the parasite on *Leptographium* sp.

Experiments were then designed to determine the concentrations of manganese and magnesium which were most effective. The control medium was 5–5 glucose-yeast extract agar. The following amounts in ml of the manganese solution (as $MnSO_4$) were added per liter: 0.5, 1, 2, 5, 10, and 20. One ml of the solution contained .01 mg of manganese. The amounts of $MgSO_4 \cdot 7H_2O$ varied from 0.5 to 2.5 g per liter.

The best medium for *G. fuscum* on *Graphium* sp. after ten days contained Mn and Mg in the smallest added amounts. The next best media were 20 ml of manganese solution added and the combination of 5 ml of manganese solution and 2.5 g of $MgSO_4 \cdot 7H_2O$ added. The parasite was absent on three media; the control, .5 ml manganese solution added, and 0.5 g $MgSO_4 \cdot 7H_2O$ added.

On *C. ulmi* the parasite was present in all media except those containing less than ten ml of the manganese solution without added $MgSO_4$. The parasite was present on *Leptographium* sp. in all media, but appeared best in the media containing both metals added in the smallest amounts. High concentrations of manganese also favored the growth of the parasite on *Ceratocystis pluriannulata* as the host.

The addition of different concentrations of microelements to 5–5 glucose-glutamic acid media did not stimulate growth of the parasite, but did stimulate synnema production of *Graphium* sp. Similar results on the lack of parasitism were also obtained using *C. ulmi* as the host. No differences in host growth were observed.

It was concluded from these results that the concentration of certain metals in the medium greatly affected the degree of parasitism, although usually there was no visible effect on the host. Manganese stimulated the growth of *G. fuscum* when added to a 5–5 glucose-yeast extract medium. A correlation between the activation or inhibition of certain enzymes and parasitism is conjectured, since the amounts of the metals required were greater than would be expected if serving as nutrients for the host.

DISCUSSION

The natural association of *G. fuscum* with *Ceratocystis* spp., suggested by earlier reports was verified in the present work. The parasite was found on *Graphium* sp. in fourteen oak trees in seven counties in West Virginia.

The mode of parasitism of *G. fuscum* is unique and only one other known fungus, *Gonatobotrys simplex* (unpublished) is similar to it. Both fungi contact their hosts by means of small specialized absorptive hyphae. No penetration of the host has been noted. These small absorptive hyphae resemble those described by Barnett and Lilly (**1958**), for *Calcarisporium parasiticum,* but lack the small buffer cell.

The possibility exists that these absorptive hyphae may function physiologically as do haustoria, although they do not penetrate the host. The clasping nature of the branches of *G. fuscum* indicates a very close union, and since connections are with young hyphae, the cell walls of host and parasite are immature, and therefore, thinner membranes separate protoplasts of the two fungi at these points of contact.

It also can be conjectured that the center of certain enzyme activities lies in these absorptive hyphae. A similar hypothesis concerning *Calcarisporium parasiticum* was proposed by Barnett and Lilly (**1958**). Manganese and magnesium stimulated growth of *G. fuscum,* suggesting that enzymes play an important role in the host-parasite relationship. Additional investigations of these absorptive hyphae are needed and they offer good opportunities for obtaining valuable information about the principles of parasitism.

A reversal of the host-parasite relationship occurred when the old aerial hyphae and conidiophores of *G. fuscum* were commonly attacked by hyphae of *Graphium* sp. The hyphae twined around *G. fuscum* and often penetrated the cells. Hawker et al. (**1957**) reported that the growth of *Pythium ultimum,* a parasite on *Allium ursinum,* was often arrested and sometimes destroyed by the host. Since *Graphium* sp. only acts in a parasitic manner after the nutrients are apparently ex-

hausted from the medium, several explanations are possible. The abundant, vigorous mycelium of *G. fuscum* may cease to grow because of the limited host growth after the exhaustion of the nutrients. *Graphium* sp., not so specific in nutritional requirements as *G. fuscum*, then attacks the large aerial parts of the latter which contain an abundance of nutrients. Another possibility is that the host, which is dependent on an exogenous supply of pyridoxine, attacks *G. fuscum* because it contains this needed vitamin. The appearance of scattered synnemata at that time suggests this latter possibility.

All species of *Ceratocystis* tested were hosts for *G. fuscum*, but some hosts supported better parasite growth than the others. Barnett and Lilly (**1958**) and Berry (**1958**) reported different degrees of parasitism on different hosts in investigations with *Calcarisporium parasiticum* and *Piptocephalis virginiana*, respectively. Host physiology is an important factor in the host-parasite complex.

Since *G. fuscum* also parasitized *Graphium* sp. and *Leptographium* sp., the possibility that they have perfect stages belonging to the genus *Ceratocystis* is suggested. A means of separating two genera both having a *Graphium* imperfect stage then might be possible by observing whether *G. fuscum* will parasitize the questionable species. Although *Petriella asymmetrica* has a *Graphium* imperfect stage, *G. fuscum* did not attack it.

Gonatobotryum fuscum ordinarily grew slower on *C. ulmi* than on other hosts, but in time the parasite grew far out from the edge of the host colony. The parasite also reacted in this manner on *Graphium* sp. when the cultures were incubated in total darkness. Light was necessary for normal synnema production with *Graphium* sp. The establishment of *G. fuscum* on *Graphium* sp., usually after seven days, resulted in the inhibition of synnema production. These results indicate a close association between the necessary nutrients and certain metabolites, catalyzed by light for synnema formation in *Graphium* sp. *Leptographium* sp., a dark fungus, was the best host for *G. fuscum*, and supported good growth of the parasite on media regarded as poor for parasitism using *C. ulmi* and *Graphium* sp. as hosts.

Butler (**1957**) pointed out that *Rhizopus* sp., *Mucor recurvus*, *Pythium debaryanum*, and *Pythium butleri*, were more susceptible to attack by *Rhizoctonia solani* in total darkness than in constant light. Nickerson and Merkel (**1953**) reported that light activated certain flavoprotein enzymes which reduced tetrazolium salts. Metal ions associated with the tetrazolium salts stabilized it against photoreduction. Since the addition of manganese to certain media otherwise unsuitable for para-

sitism stimulated the growth of *G. fuscum* on its host, it is possible that the metal stabilized certain unknown materials against reduction.

The nutrition of the host has been demonstrated to affect the degree of parasitism. It is possible that different carbon-nitrogen ratios and concentrations of microelements in the medium influence the production of certain enzymes which in turn affect the degree of parasitism. It is also possible that greater parasitism under some conditions reflects a greater supply of amino-acids or other required nutrients within the host cells.

SUMMARY

Gonatobotryum fuscum was shown to be a mycoparasite of the balanced or biotrophic type. The spores of *G. fuscum* germinated on several different media, but in the absence of a living host, the mycelium soon ceased to grow. Old aerial hyphae and conidiophores of *G. fuscum* were commonly parasitized by a host fungus, *Graphium* sp. in what is termed a reversal of parasitism.

The host range of *G. fuscum* includes fungi belonging to the genus *Ceratocystis* or related species of imperfect fungi. The parasite reacted differently on different hosts.

Light and temperature affected the degree of parasitism of *G. fuscum* on several hosts. Total darkness at 20° C was optimum for growth of the parasite. *G. fuscum* did not grow on any host above 30°, but the spores were not killed at this temperature. Although darkness stimulated parasitism, it also inhibited synnema formation of *Graphium* sp. The establishment of the parasite on this host also inhibited synnema formation.

G. fuscum grew best on its hosts in media containing a relatively high amount of carbon and a low amount of nitrogen. It grew only on *Leptographium* sp. in a medium high in nitrogen.

Growth of the parasite was stimulated when manganese was added to media otherwise not supporting good growth of *G. fuscum* on its hosts.

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THE NATURE OF PRODUCTION OF THE GLYOXYLATE PATHWAY ENZYMES IN GERMINATING SPORES OF PENICILLIUM OXALICUM (F–56) [1, 2]

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(with 2 figures)

The glyoxylate pathway, which has been found in certain bacteria, fungi, and higher plants, is a pathway for net-synthesis of carbon skeleton from C_2 compounds. These carbons enter the citric acid cycle as acetyl-CoA, by condensing with oxaloacetic acid to give rise to citric acid which is in turn converted to isocitric acid. Instead of being further metabolized via the citric acid cycle, the isocitric acid is cleaved by the enzyme isocitritase to yield glyoxylic acid and succinic acid. The glyoxylic acid then condenses with acetyl-CoA to form malic acid in the presence of the second enzyme malate synthetase (**6**, **7**, **9**, **13**). Thus by so bypassing the citric acid cycle via the glyoxylate pathway, the loss of carbons as carbon dioxide is avoided. Evidence reported so far indicates that the enzymes isocitritase and malate synthetase are produced adaptively, in the presence of acetate (**8**, **10**). The current studies were undertaken to determine if the glyoxylate pathway enzymes were contained in the spores of *Penicillium oxalicum* Currie & Thom and, if present, whether they were constitutive or adaptive.

MATERIALS AND METHODS

P. oxalicum spores were produced by growing the fungus in Fernbach culture flasks of 2.8 liter capacity containing 300 ml of V-8 medium.[3] Five ml of spore suspension of *P. oxalicum* in sterile distilled water, giving 30% transmission in a densitometer, were pipetted into each flask

[1] University of Illinois soil isolate, 1952.

[2] Germinating spore includes the spore and the germ-tube.

[3] V-8 juice, 200 ml; calcium carbonate, 3.0 g; agar, 20.0 g; distilled water to make 1000 ml. Medium sterilized by autoclaving at 15 pounds pressure for 15 minutes.

and the flasks swirled to distribute the spores evenly on the agar surface, and the flasks were incubated at 26.7° C for 7 days. The cotton plug was removed and the flask inverted over a sterile Petri plate and tapped gently to detach the spores which were collected in the Petri plate. More than 10 g of spores were collected from 15 flasks. The spores were transferred to a culture tube, corked and stored at 2° C.

Since it was difficult to break the spores in sufficient numbers to produce a good cell-free enzyme preparation, the spores were germinated for 24 hours and then the enzyme preparation was made. The spores also needed an external source of carbon for germination. Glucose supported germination very well, but acetate not as efficiently. Pretreating spores in a glucose solution for 4 hours later gave much better germination in acetate solution.

Four liter flasks, each containing 500 ml of 1% glucose, yeast-nitrogen-base medium (YNB) (**12**), and 1:10,000 vatsol as wetting agent, were autoclaved at 15 lb pressure for 15 minutes. One-half gram of *P. oxalicum* spores was added to each flask and the flasks were put on a rotary shaker. After four hours, which allowed some swelling but no spore germination, two flasks were removed, and the spores from each flask were washed three times with phosphate buffer, pH 4.5. These activated spores were then added to two 1 liter flasks, each containing 1% potassium acetate and YNB medium. After 24 hours when germ tube length averaged 40 μ, all four flasks were removed from the shaker. The germinated spores in the 2 flasks containing glucose and the 2 containing acetate were pooled separately and each collected by centrifugation. Each batch was washed 3 times with phosphate buffer solution at pH 6.0, by suspending the spores in buffer, centrifuging, and finally suspending each in 100 ml of the buffer. Eighty ml of these suspensions were sonicated in a 10 K.C. Raytheon Sonic Oscillator for 20 minutes. The sonicated suspensions were centrifuged at 4° and 1500 × G for 20 minutes and the cell free supernatant liquids saved. The solutions from the glucose-germinated and the acetate-germinated spore material were individually dialyzed against three changes of phosphate buffer, pH 6.0, over a period of 18 hours at 4° C to remove any substrates and co-factors like DPN, TPN, CoA, ATP, etc. which would otherwise interfere in the controlling of the experimental reaction steps.

The presence of the glyoxylate by-pass enzymes in these two preparations was determined by a dynamic assay and also by assaying for the products formed by these two enzymes on catalysis of specific substrates.

The method of Dixon and Kornberg (**1**) was used in the dynamic assay. The assay of isocitritase, which catalyzes the reaction, isocitrate

$\rightleftharpoons$ succinate + glyoxylate, depends on the measurement of the rate of increase of optical density at 324 mμ, due to the formation of the glyoxylic acid phenylhydrazone from glyoxylate and phenylhydrazine. Three ml of reaction mixture contained 200 μmoles potassium phosphate, pH 6.85; 15 μmoles of $MgCl_2$; 10 μmoles of phenylhydrazine HCl; 6 μmoles cysteine HCl; 0.5 ml of the enzyme preparation and water. The reaction was started by the addition of 5 μmoles of potassium (L) isocitrate in a cuvette. The changes in O.D. were recorded using a Beckman DU Spectrophotometer. Readings were taken once every minute.

For the assay of malate synthetase, which catalyzes the reaction, acetyl-CoA + glyoxylate → malate, the method of Stadtman (**11**), as used by Dixon and Kornberg (**1**), was used with slight modification. The acetyl-CoA has an absorption maximum at 232 mμ. Starting with acetate ATP and CoA, the formation of the thiol-ester bond of acetyl-CoA, which is catalyzed by the acetate activating enzyme, was followed by observing the increase in O.D. at 232 mμ on the addition of the enzyme preparation. Then, the decrease in the O.D. at the same wavelength due to breakage of the thiol-ester bond of acetyl-CoA with the addition of glyoxylate, when malate was formed, indicated the presence of malate synthetase. The reaction system contained in 3.0 ml, 2750 μmoles of potassium phosphate pH 7.0; 10 μmoles $MgCl_2$; 2 μmoles of potassium acetate; 2 μmoles of CoA; 5 μmoles ATP; 0.05 ml enzyme preparation, 4 μmoles sodium glyoxylate and water. The changes in O.D. were recorded on a recording spectrophotometer. In the second step of the reaction, the breakage of the thiol-ester bond of acetyl-CoA, the ΔE_{232} was recorded for one minute to see if any deacylase was present, which would hydrolyze the thiol-ester bond of acetyl-CoA before the glyoxylate was added.

The method of Kornberg and Madsen (**4**) was used with slight modifications for the static assay with acetate-1-C^{14} as the isotope tracer. To prevent the operation of the T.C.A. cycle which would also form malate, a dialyzed enzyme preparation, which exhibited no dehydrogenase activity, was used. The experiment was run under nitrogen to eliminate any further changes of the operation of the T.C.A. cycle. Warburg vessels shaken in the constant temperature bath were used for the reactions. The experimental design is given under TABLE I.

The flasks were flushed with N_2 for 10 minutes, and equilibrated in the temperature bath for 15 minutes, before the ATP was tipped in at zero time. At the end of 60 minutes, the reaction in each flask was stopped by adding 3.0 ml of hot absolute alcohol (80° C). After leaving the flasks at 50° for 15 minutes the flask contents were drained into a

centrifuge tube, spun at 1500 × G for 20 minutes, and the supernatant liquid saved. The precipitate was washed with 1 ml of 20% ethanol, centrifuged, and the two supernatants were pooled. This solution was evaporated to dryness at 60° C under a gentle stream of sulphuric acid-washed nitrogen. The residue was taken up in 1.0 ml of 6N hydrochloric acid and again evaporated to dryness at 100° C in the same manner. The dried material was taken up in 0.5 ml of 20% ethanol. Two hundred fifty μl of this solution was applied on a Whatman no. 3 paper for descending two dimensional chromatography. The paper was equilibrated and chromatographed in a phenol:formic acid system (500 g

TABLE I

FORMATION OF MALATE AND SUCCINATE VIA THE GLYOXYLATE CYCLE BY THE A.G. AND G.G. PREPARATIONS*

Flask contents		Flask set				
		A	B	C	D	E
Reactants (*μmoles*)						
Acetate		30	30	30	—	150
Glyoxylate		—	30	—	—	—
Isocitrate		—	—	30	50	—
Oxalsacetate		—	—	—	—	50
(μcuries—acetate-1-C^{14})		(10)	(10)	(20)	—	(40)
Products (*μmoles*)						
Malate	—A.G. Prep	—	1.90	1.75	—	1.60
	—G.G. Prep	—	0.98	0.94	—	0.50
Succinate**	—A.G. Prep	—	+	—	+	2.1
	—G.G. Prep	—	+	—	+	0.5

* In addition to the reactants, each flask contained the following: Potassium phosphate buffer, pH 7.0, 300 μmoles; magnesium chloride, 30 μmoles; glutathione, 30 μmoles; coenzyme A, 0.5 μmoles; ATP, 40 μmoles; A.G. preparation, 0.25 mg protein; or G.G. preparation, 0.5 mg protein. The experiment was conducted at 29.6° under N_2 in a Warburg Apparatus for 60 min.

** "+" indicates that nonradioactive succinate was found.

phenol:13 ml of 90% formic acid:167 ml water). The paper was then air dried for 24 hours, equilibrated and chromatographed in the second system viz., 1:1 n-butanol:priopionic acid (1246 ml n-butanol + 84 ml water; 620 ml proprionic acid + 790 ml water). The chromatogram was air dried for 24 hours. Similar chromatograms of the standards, malate and succinate, were sprayed with 0.04% bromophenol blue in 95% ethanol adjusted to pH 8.0 with sodium hydroxide. Using the standards spots as markers, strips were cut from the chromatograms containing the radioactive material and the radioactivities of the malate and succinate were counted in a model PCR-105 strip paper counter. The

total radioactivity in each spot was determined by measuring the area under the recorded radioactivity peak for each spot and determining the total activity from the standard graph. The activity of the acetate used was measured by counting a sample of the acetate-1-C^{14} solution used in a Scintillation Counter. From the values obtained by strip counting, the amount of malate formed via the glyoxylate cycle was calculated.

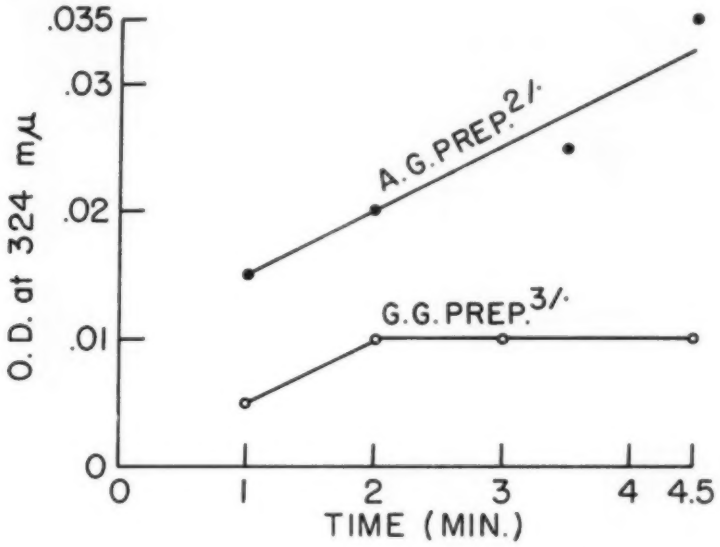

FIG. 1. A comparison of isocitritase activity in acetate- and glucose-germinated preparations. Three ml of reaction mixture contained 200 μmoles potassium phosphate, pH 6.85; 15 μmoles $MgCl_2$; 10 μmoles phenyl hydrazine HCl; 6 μmoles cysteine HCl; 0.5 ml enzyme preparation and water. Reaction started by the addition of 5 μmoles potassium isocitrate.

2/· Acetate-germinated preparation 0.5 mg protein per ml.

3/· Glucose-germinated preparation 1.0 mg protein per ml.

Protein analyses were done by the standard Biuret method. Glucose analyses were made manometrically using glucose oxidase and catalase (3). Dry weights were recorded by drying the samples at 80° C for 48 hours and weighing.

RESULTS

The activity of the enzyme isocitritase in acetate-germinated preparation (A. G. prep.), as measured by the increase in O.D. at 324 mμ, gave

a typical reaction. The increase in the O.D. was linear for about 5 minutes (FIG. 1). The glucose-germinated preparation (G. G. prep.), however, did not give a characteristic increase in the O.D. at 324 mμ.

The presence of the enzyme, malate synthetase, in the A. G. prep. was shown by the decrease in the ΔE_{232} on the addition of the glyoxylate to the cuvette. The increase in O.D. on the addition of the enzyme prepa-

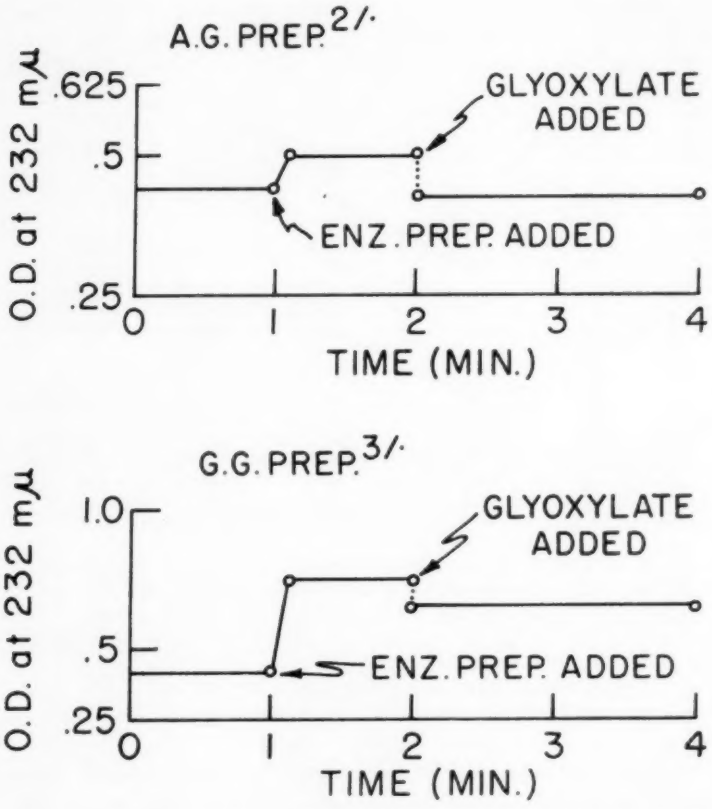

FIG. 2. A comparison of malate synthetase activity in acetate- and glucose-germinated preparations. The reaction mixture contained in 3.0 ml, 2750 μmoles potassium phosphate, pH 7.0; 10 μmoles $MgCl_2$; 2 μmoles potassium acetate; 2 μmoles CoA; 5 μmoles ATP; 0.05 ml enzyme preparation; 4 μmoles sodium glyoxylate and water.

2/· Acetate-germinated preparation 0.5 mg protein per ml.

3/· Glucose-germinated preparation 1.0 mg protein per ml.

ration to the cuvette containing ATP, acetate and CoA shows the presence of acetate activating enzyme system which catalyzes the formation of acetyl-CoA. The lack of decrease in O.D. prior to the addition of glyoxylate indicates the absence of acetyl-CoA deacylase in preparation. The G. G. prep. showed approximately half the malate synthetase activity and the sharp increase in O.D. at 232 mμ on the addition of the enzyme preparation might be due to the translucency of the preparation itself (FIG. 2).

Data in TABLE I show the absence of succinate and malate in A, malate in B, and succinate in C. Thus, it is evident that the citric acid cycle was not operative under these experimental conditions. The presence of isocitritase in both glucose- and acetate-germinated preparations was proved by the formation of succinate in B, D, E, and malate in D.

TABLE II

INCREASE IN DRY WEIGHT AND PROTEIN, GLUCOSE UTILIZATION, AND pH CHANGES IN THE CULTURE-MEDIUM DURING GERMINATION OF PENICILLIUM OXALICUM SPORES

Time in hours	mg dry wt/ml	mg protein/ml*	mg glucose left /ml of culture medium	pH
0	0.44	0.22	10	4.50
6	0.57	0.30	8.75	3.50
12	1.25	0.62	7.40	2.70
18	3.52	1.51	2.70	2.45
24	5.54	2.44	0.28	2.19

* Protein analyzed by the micro-Kjeldahl method.

Likewise, the presence of malate synthetase in both the preparations is established by the formation of malate in C, D, and E. Despite the lower protein content of A. G. prep. it formed more malate and succinate than the G. G. prep., indicating a higher content of glyoxylate cycle enzymes.

The determination of protein synthesis during germination was made because any large increase in protein would dilute out the glyoxylate cycle enzymes if more were not synthesized during germination. Over the 24 hour period, protein increased over tenfold (TABLE II). Changes in pH were recorded to determine whether acids were produced which might induce the glyoxylate cycle. The pH of the culture fluid dropped from 4.5 to 2.19 and traces of acetic acid in the culture fluid were identified chromatographically but only at the end of 24 hours. Glucose utilization was determined because glucose is believed to inhibit the induction of glyoxylate cycle enzymes. It can be seen that, even though

most of the glucose was consumed, small quantities were still present at the end of 24 hours.

DISCUSSION

From the data presented it is evident that the two glyoxylate by-pass enzymes are present in both acetate-germinated and glucose-germinated spores of *P. oxalicum*, though the enzyme content is certainly higher in A. G. prep. The very presence of these enzymes in the glucose germinated preparation tempts one to postulate that the enzymes isocitritase and malate synthetase are constitutive in germinating spores of *P. oxalicum*. This is contrary to the findings of other investigators in other organisms (**8**, **10**). Reeves and Ajl (**8**) have recently shown that these two enzymes are adaptive in *E. coli* strain E_{26}.

The data in our experiments indicate that acetate stimulated the production of the glyoxylate cycle enzymes. Traces of acetate were found, however, in the culture fluid of glucose grown spores at the end of 24 hours but none at 18 hours. It is conceivable that one could build a thesis that the accumulation of acetate is the key to the adaptive formation of glyoxylate cycle enzymes in the spores germinated with glucose. Such an induction is postulated by Gollakota and Halvorson (**2**) in the sporulation of *Bacillus cereus*. On the other hand, these investigators found that this adaptive process was inhibited as long as glucose was found in the culture fluid. Under our conditions, glucose was still present in the culture fluid when the two enzymes were found to be present. Therefore, the glyoxylate cycle enzymes could not have been induced by the accumulating acetate in the presence of glucose in the culture medium. The alternate then is that these enzymes are constitutive in germinating spores of *P. oxalicum*.

The tenfold increase in protein content of the glucose-germinated spores, within 24 hours of germination, precludes the possibility that the glyoxylate by-pass enzymes, which were found in the glucose-germinated preparation, were the induced residual enzymes that were originally enclosed in the spores during spore-formation. The tenfold protein increase would have diluted out any such enzymes, if more of the enzymes were not produced as the spore germinated.

SUMMARY

Data suggest the presence of an operative glyoxylate cycle in germinating spores of *P. oxalicum*. The two enzymes, isocitritase and malate synthetase, were present in cell free preparation from spores germinated with either glucose or acetate as substrates. The concentrations

of both enzymes were higher in acetate-germinated than in glucose-germinated spores. At the end of 24 hours of germination with glucose as substrate, traces of acetate could be identified in the culture fluid as well as glucose. From these facts it is concluded that the two glyoxylate-cycle enzymes could be constitutive in germinating spores of *P. oxalicum*.

ACKNOWLEDGMENT

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CYTOLOGY OF PUCCINIA SORGHI [1]

M. S. Pavgi, D. C. Cooper, and J. G. Dickson

(with 41 figures)

The genetic analysis of pathogenic variability in *Puccinia sorghi* Schw. was expanded to include a comprehensive examination of nuclear and chromosomal behavior during nuclear fusion and subsequent meiosis and mitosis. Knowledge concerning the regularity of chromosome behavior during the course of these events is essential for a rational interpretation of genetic data on variability.

The teliospore as well as the basidium and the sporidia resulting from its germination provide the key structures for a study of nuclear and chromosomal behavior. The dicaryotic phase of the life cycle in this group of fungi terminates with nuclear fusion in the cells of the teliospore. Information on the regularity of consummation of these fusions in breeding stocks such as inbred lines is relevant in estimating sterility. The developing basidium is the site of meiosis leading to the new haploid phase. A further check on chromosome behavior in the haploid phase is possible during germination of the sporidia. These processes, since they occur in structures exterior to the host, are amenable to manipulation and cytological study.

A comparison of nuclear behavior within the urediospore lines employed in investigations of variability in pathogenicity and serology of *P. sorghi* (**4**) is reported in the present paper. The analysis covers the nuclear fusion which produces the diploid nucleus, chromosome behavior during meiosis and mitosis, sporidial formation and subsequent germination. On the basis of preliminary data on inbred lines, nuclear fusion to initiate the diploid phase occurs with decreasing frequency following continued inbreeding. The resulting sterility plays an important role in the regulation of variability extremes. Therefore, chromosome behavior during meiosis and subsequent mitosis in the fertile lines should be

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regular. The mechanism conditioning variability in these fertile recombinants, therefore, rests largely in the reassortment of hereditary units carried by the chromosomes.

McGinnis (7, 8, 9) has reported the haploid chromosome number for several species of *Puccinia* including *P. sorghi*. He suggests a possible bearing of chromosome number within the genus to polyploidy and discusses its effect on pathogenicity and sequential expansion in host range (9). The present knowledge of these relationships is too meager to warrant any definite conclusions although the presence of a common antigen in the species parasitic on the grasses suggests a common ancestry (4). Irregularities in chromosome behavior and number in the associated nuclei within heterocaryotic cells in vegetative hyphae of *Helminthosporium turcicum* Pass. and their perpetuation in the imperfect stage have been associated directly with variability (6). Nuclear exchange through frequent anastomoses between hyphal cells of different clones results in further variation and its perpetuation in the imperfect stage. In contrast to the above mechanisms which foster variation, the role of sterility in the sexual stage whereby many of these irregularities are eliminated from the nuclear complement becomes equally significant in regulating variability.

MATERIALS AND METHODS

Telia of *Puccinia sorghi* lines 1–20, 2–22, and 3–32 produced under greenhouse conditions on the susceptible inbred corn line P39 were overwintered outdoors and stored at low temperature. Alternate freezing and thawing during early spring appeared to be conducive to spore germination. Teliospores were germinated on slides by alternate wetting and drying (16, 17) and incubating at 17° C. Moisture was controlled in order to prevent an accumulation of excess water immediately adjacent to or over the teliospores.

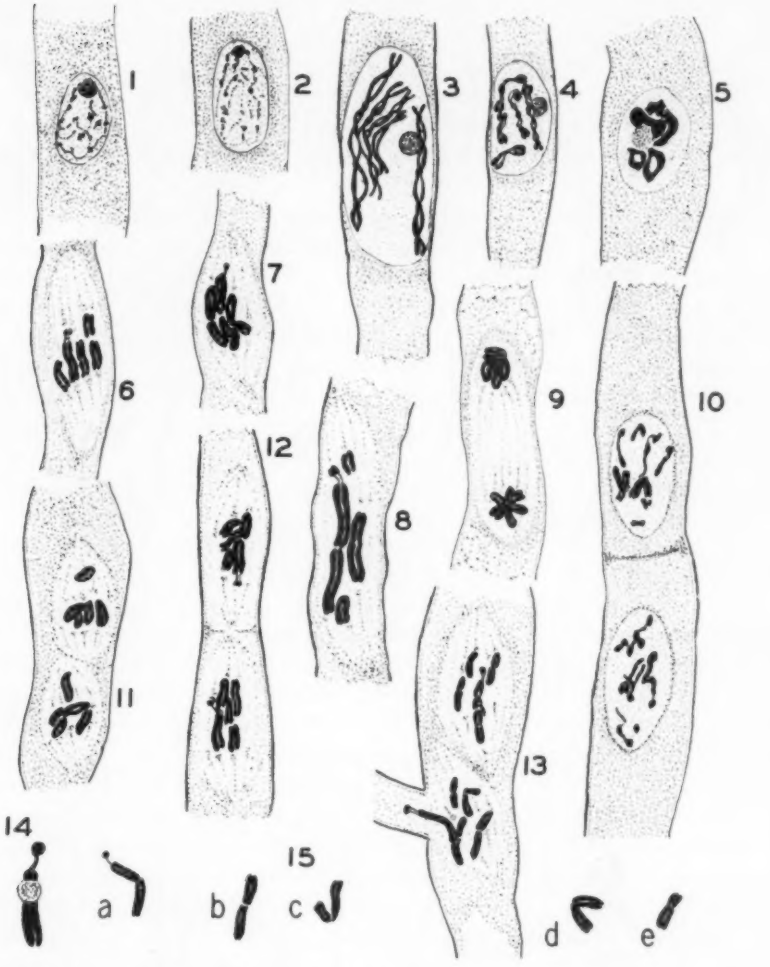
Germinating teliospores at varying stages of development were fixed, *in situ*, for one hour in modified Carnoy's fluid (3 parts 95% ethyl alcohol, 2 parts chloroform and 1 part glacial acetic acid) and then transferred to 70% alcohol. The slides bearing the spores were passed through a descending series of 4 concentrations of alcohol to water and then treated with a 0.1% aqueous solution of diastase for 1 hour. The accumulated granular storage products in the cytoplasm are rendered soluble by the diastase. After washing in water they were passed through an ascending series of alcohols to 70% and the material was stained in 1% aceto-carmine. Some were stained in Heidenhain's iron

alum haematoxylin for comparative purposes. The Feulgen staining technique proved to be unsatisfactory since much of the material was dislodged during the procedure.

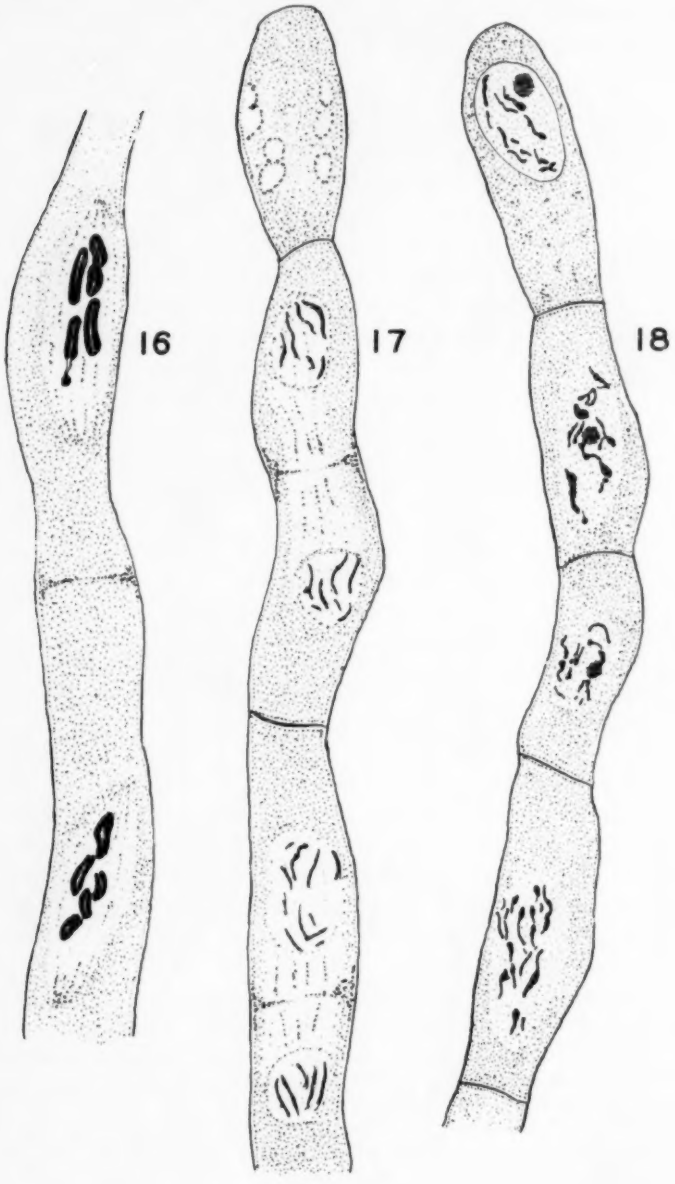
OBSERVATIONS

The occurrence of nuclear fusion in the cells of the teliospore, the termination of the dicaryotic condition, apparently is associated with the pedigree of the line and the physiological maturity of the teliospore. Such fusion generally occurs before overwintering although not infrequently it takes place immediately before germination. Preliminary studies of teliospores from certain inbred lines where aecial infections are unsuccessful reveal the absence of diploid nuclei. During the process of fusion the 2 nuclei become closely appressed, the contacting surfaces of the nuclear membranes dissociate and the 2 nucleoli eventually fuse. Persistence of the 2 nucleoli through the prophase has been reported in *Puccinia malvacearum* Bert. (**1**) and in species of *Coleosporium* (**10, 12, 14**).

Early in teliospore germination, the diploid nucleus and the associated cytoplasm move through the distended germ-pore as the stout promycelium or basidium emerges (FIG. 1). The nucleus expands during interphase (FIG. 2) and meiosis I proceeds rapidly under optimum moisture and aeration. The nucleus at interphase possesses a chromatin reticulum and a large nucleolus. The nucleus soon enters into prophase. Observations of nuclei in a large number of basidia indicate several transitional stages, but no typical leptotene stage. Olive (**12**) and Sanwal (**14**) considered prefusion nuclei as equivalent to leptotene. The morphologically-distinct homologues pair lengthwise and synapse at several points during zygotene. Synapsis of nucleolus-organizers and fusion of the nucleoli could not be determined precisely, but the large fusion nucleolus is invariably associated with the nucleolus-organizer throughout prophase. Several early diplotene stages occur where the long and fibrillar bivalents exhibit intertwining coils (FIG. 3). The chromosomes approaching this stage become thin and fibrilose instead of undergoing the initial condensation as in higher plants. This resembles the "phase of elongation" described by Olive (**12**). The 4 chromatids never become distinctly resolved although the 2 homologues of each bivalent are distinct. The chromosomes are conspicuously beaded along their entire length. Details of the chromonemata could not be resolved due to insufficient differentiation. Three to 5 chiasmata are formed between the chromosomes of each bivalent depending on the



FIGS. 1–15. Meiotic nuclear division in the germinating teliospores of *Puccinia sorghi* (×2700). FIG. 1. Diploid nucleus in the basidium. FIG. 2. Expanded diploid nucleus. FIG. 3. Early diplotene. FIG. 4. Diplotene at an advanced stage. FIG. 5. Diakinesis. FIGS. 6–8. Stages of chromosome orientation on the metaphase I plate. FIG. 9. Late anaphase I. FIG. 10. Prophase II. FIGS. 11–13. Metaphase II. Stages in chromosome orientation. FIG. 14. SAT-Chromosome with a prominent centromere. FIG. 15 (a to e). Five chromosomes of the haploid complement showing some morphological features.



FIGS. 16–18.

chromosome length (FIG. 4). The thick, condensed chromosomes possess widely stretched loops at diakinesis (FIG. 5). Relatively few reports on the sequence in prophase have appeared in the literature. Berliner (**3**) and Payak (**13**) have described diplotene and chiasmata formation in *Gymnosporangium clavipes* Cooke & Peck and *Scopella gentilis* (Syd.) Mundk. & Thirum., respectively, other reports being indefinite.

The metaphase spindle, often indistinct with ill-defined fibers, becomes oriented parallel to the long axis of the basidium. No astral rays emanating from the poles or polar caps are discernible even in materials stained with Heidenhain's haematoxylin. They have been consistently reported in the species of *Coleosporium* (**5**, **10**, **12**, **14**) and *Thekopsora hydrangeae* Magn. (**11**). Deeply stained and refractile particulate bodies slightly offset from the poles are occasionally present and may possibly represent the centrioles.

The spindle is large enough to accommodate initially all the chromosomes in the complement at the plate. The chromosome pairs are more or less regularly orientated (FIGS. 6 to 8) and not compacted or distributed over the spindle as in some other species (**9**). The spindle attachment fibers are evident at the centromeres, depending on the chromosome orientation. Anaphase is initiated in the continuum of the orientation process. The chromosomes of the bivalents separate as the spindle elongates and the 2 sets move to the poles (FIG. 9). In the early anaphase they are distinct, but soon become massed into aggregates and merge into telophase with no indication of membrane formation. Cytokinesis proceeds by furrowing. These stages occur in rapid sequence and are not frequently observed.

The interphase also is of short duration. Transition from the partially oriented chromatin reticulum to metaphase II passes through an evanescent prophase (FIG. 10). Interphase or near-prophase stages are occasionally evident. The nuclear membrane is not well defined and the chromosomes with deeply stained chromonemata are only partially differentiated during these phases.

Meiosis II is initiated with the synchronous organization of 2 spindles and orientation of the chromosomes on each spindle (FIGS. 11 to 13, 16). The 5 chromosomes representing the hapoid complement for *P. sorghi*

FIGS. 16–18. Meiotic nuclear division in the germinating teliospores of *Puccinia sorghi* (× 2700). FIG. 16. Metaphase II in early stage. FIG. 17. Telophase II. Note the enucleate terminal cell. FIG. 18. Nuclear organization in basipetal succession.

are clearly evident at this stage. This observation is at variance with those of Savile (**15**) who reported 4 chromosomes and McGinnis (**9**) who reported 6 chromosomes for this pathogen. The morphology of the chromosomes was determined during the meiotic stages (FIGS. 8, 14, 15).

Morphology of chromosomes: The 5-chromosome complement at metaphase I occurs consistently. Two of these are long and nearly equal in size, 2 are medium and similar in size and 1 is short (FIG. 15). Evidence of heterochromatic regions and the nature of spiralization is sparse due to the limitations in use of the Feulgen reaction and other procedures. Chromosome 1, the longest in the complement measures 6 to 7 μ and has a submedian centromere at the constricted zone dividing it into 2 unequal arms (FIG. 15a). Two characteristic and distinguishing features are evident; a secondary terminal constriction bearing a small round knob—the trabant or satellite—supported over a thin chromatin thread and the interstitial nucleolus-organizing region (FIG. 14). The nucleolus is associated with the short arm of the chromosome. The reports of SAT-chromosomes have been uncommon in the Uredinales and perhaps in other fungi. Such a chromosome has been described for *Scopella gentilis* (**13**). The other chromosomes present few distinguishing features beyond the general morphological characters. Chromosome 2 with a submedian centromere and 2 unequal arms is 6 μ in length (FIG. 15b). Chromosomes 3 and 4 are 5 μ in length with median centromeres (FIG. 15c, d). Chromosome 5, which is the short member of the complement, is 2.5 μ long with a median centromere (FIG. 15e).

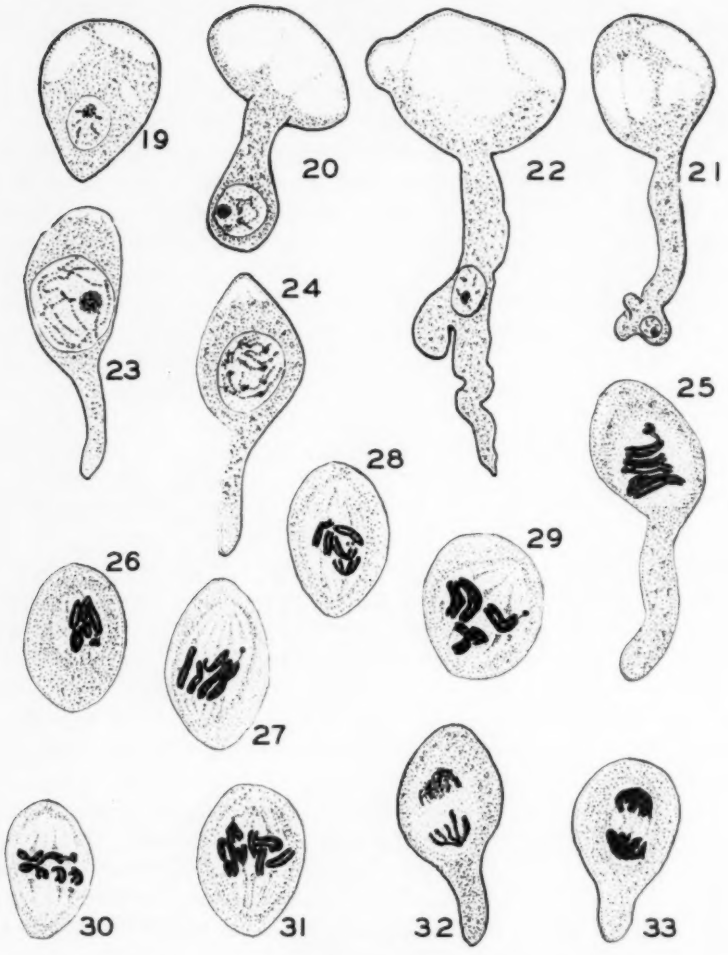
Deviations in the orientation of the spindle or size and shape of the chromosomes are not evident at metaphase. The presence of chiasmata and crossovers in prophase I show how segregation for certain characters may occur in the second meiotic division. Anaphase and telophase of the second division follow in quick succession (FIGS. 17, 18), and the members of each dyad separate and move to opposite poles of the spindle. Development of transverse septa begins with the appearance of phragmoplast granules during metaphase II or early anaphase II, and the septa develop during telophase. Nucleoli appear as glistening globules in the aggregated chromatin, apparently associated with the nucleolus-organizers after the 4 daughter nuclei become enclosed in the new nuclear membranes. There is no evidence of chromosome bridges and unequal divisions of the chromosome complement during meiosis. The meiotic division in *P. sorghi* appears to be regular and in general conformity with data on the recombination of heritable units in fully compatible

unions. This perhaps is to be expected, as incompatibility apparently becomes eliminated by the failure of nuclear fusion in the teliospore and such spores not germinating.

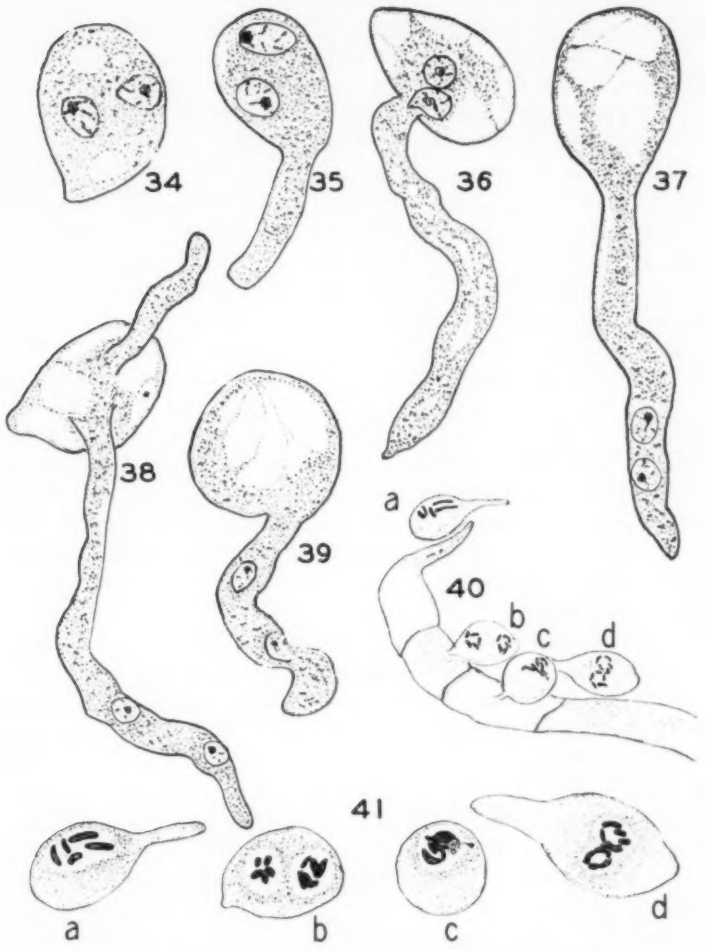
Basidiospore Cytology: Development, maturation and ejection of basidiospores proceeds in basipetal succession. The nucleus and cytoplasm migrate into the young vesicle, and some cytoplasm usually remains in the sterigma. Precocious mitosis of the haploid nucleus occasionally occurs in the basidial cell. Several types of aberrations in the germination of basidium are encountered under high moisture conditions which lead principally to 1) delayed or inhibited nuclear divisions and septation of the basidium, 2) failure in normal development and number of basidiospores and 3) a strong tendency to sterigmatal branching. These appear to be directly associated with the moisture film and the orientation and growth of the structures as they emerge from the water medium.

Mature basidiospores are typically pyriform to oval, hyaline, thin-walled (FIGS. 19, 34) and measure 9 to 14 × 7 to 11 μ. They germinate rapidly *in vitro* by the initiation of 1 or 2 slender germ tubes. Nonpolarity or lack of predetermined germination sites is evident since the germ tubes develop at random from the surface of the spore. No budding or secondary spore formation such as has been reported in *Coleosporium* (**10, 12**) and *Gymnosporangium* (**3**) is observed. The elongating germ tube dilates into a vesicle—the appressorium—and 1 to 2 nuclei move into it (FIGS. 21, 22, 35 to 39). Occasionally germination occurs without mitosis and the single haploid nucleus migrates into the germ tube (FIGS. 20–22). Two mitoses resulting in 4 nuclei occurred in several basidiospores.

The mitotic division occurs in the basidiospore at or prior to germination. It was difficult to determine precisely whether mitosis precedes germination or *vice versa*. Maturity of the spore, detachment from the sterigma and free moisture all influence nuclear behavior as well as germination. In normal sequence of mitosis, the interphase nucleus expands and elongate chromosomes are formed (FIG. 23). The nucleolus disappears during prophase (FIG. 24). The nuclear membrane dissociates and metaphase proceeds rapidly. The 5 chromosomes are evident here as in the earlier meiosis. The chromosomes are identical morphologically with those observed earlier. They move onto the spindle, become oriented at the equatorial plate (FIGS. 25 to 30), split longitudinally and the 2 sets move poleward (FIG. 31). The spindles are short, relatively small and become oriented at random within the spore. No astral rays or centrioles are discernible at the poles. The chromo-



FIGS. 19–33. Germination and mitotic nuclear division in basidiospores of *Puccinia sorghi* (×2700). FIG. 19. Mature uninucleate basidiospore. FIG. 20. Single nucleus in the germ tube of a germinating basidiospore. FIGS. 21, 22. Development of appressorium and infection hypha on the germ tube and nuclear migration. FIG. 23. Expanded nucleus in a germinating basidiospore. FIG. 24. Prophase of mitosis. FIGS. 25–30. Metaphase in successive stages of chromosome orientation. FIG. 31. Early anaphase. FIG. 32. Late anaphase. FIG. 33. Telophase.



FIGS. 34–41. Germination and mitotic nuclear division in basidiospores of *Puccinia sorghi* (× 2700). FIG. 34. Mature binucleate basidiospore. FIGS. 35–38. Germination of basidiospore and nuclear migration in the germ tube. Note the smaller enucleate germ tube in FIG. 38. FIG. 39. Development of appressorium by the germ tube. FIG. 40. Mitotic divisions at metaphase in basidiospores before and after expulsion (× 1375). FIG. 41. Basidiospores in FIG. 40 enlarged. a, c. Metaphase of first mitosis. b, d. Second mitosis in ungerminated basidiospores.

somes remain fairly distinct through late anaphase (FIG. 32) or early telophase (FIG. 33) and later aggregate into a dense mass at each pole.

The daughter nuclei after a short interphase infrequently enter into a second mitosis (FIG. 40). The divisions are usually synchronous. The chromosomes become oriented obliquely and are more or less elongated during their movement to the plate (FIG. 41c). A continuous spireme was reported by McGinnis (7) in *Puccinia graminis* Pers. Polar views of the metaphase plates show ringlike configurations (FIG. 41b, d). The chromosomes are, however, individually distinct and no intact ring is evident such as was observed in *Puccinia malvacearum* (1). Degeneration of 1 or more nuclei or extrusion of chromatin in the cytoplasm as reported in species of *Coleosporium* (2) is not evident in the basidiospore nuclei of *P. sorghi*.

DISCUSSION AND SUMMARY

Germination of teliospores of *Puccinia sorghi* appears dependent upon nuclear fusion to initiate the diploid nucleus, and upon physiological maturity and the indirect and direct influence of environment. Preliminary data indicate that in some inbred dicaryons nuclear fusion is not accomplished. This perhaps represents a method related to sterility in the sexual stage that results in the elimination of extremes in variability. More extensive search for irregularities in meiotic divisions and resulting sterility due to incomplete haploid complements in basidial and sporidial production might well contribute to the knowledge of the influence of this phenomenon in regulating variability. In the lines studied excessive moisture invariably causes aberrations in the mode of germination and sporidial formation. However, under optimal moisture and temperature conditions, the sequence of meiotic processes and chromosome behavior appears to be regular.

The cytological evidence presented indicates that the sequence of stages in the nuclear divisions is regular. The chromosomes undergo the processes of synapsis of homologues, chiasma formation, anaphase, disjunction and segregation of chromatids in typical fashion. The time and period of synapsis require more critical study including more diverse lines in relation to synaptic attraction and location of synaptic sites on the chromosomes for these are important criteria with a direct bearing on the inheritance behavior of the pathogen.

The presence of centrioles and astray rays at the poles in the rust fungi remains in an indeterminate state. They have been consistently reported in *Coleosporium*, but their presence in species of other genera

is uncertain. Their presence and function have been demonstrated in the Ascomycetes such as *Neurospora*. Improved staining procedures may possibly elucidate their general presence and function in the rust fungi.

Morphologically, 2 chromosomes of the complement in *P. sorghi* are distinctive and the remaining 3 appear relatively similar in shape, length and position of centromere. The presence or absence of heterochromatin and the chromomere patterns are characteristic for each chromosome although no critical attempt was made to characterize the chromosomes on this basis.

Studies in the genetic analysis of pathogenic variability in the Uredinales suffer from the absence of a suitable technique for ready tetrad analysis. Dicaryons, heterogenous for compatibility and pathogenicity factors in a basidiospore, the parasitic nature of the haploid generation and the role of compatibility (+ and −) factors in the spermatia and receptive hyphae in the initiation of the new dicaryon recombination and resulting aeciospore production are involved. A genetic analysis for pathogenicity to corn by means of aecial sibling recombinations on *Oxalis* originating from single telial lines appears logical on the basis of the cytogenic evidence presented.

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SPORANGIAL DEVELOPMENT IN LAMPRODERMA ARCYRIONEMA

IAN K. ROSS

(WITH 16 FIGURES)

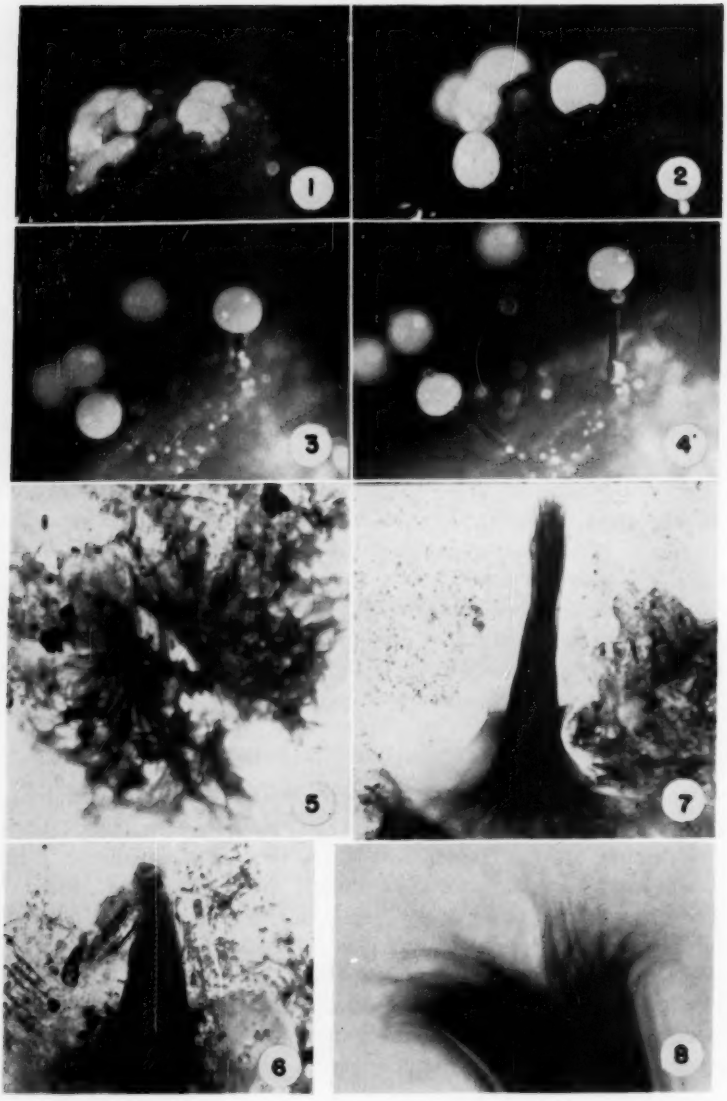
The regular procession of events which occurs during the development and maturation of sporangia in the Myxomycetes makes these organisms excellent subjects for the study of differentiation. The members of the Stemonitaceae are especially favorable for observing the differentiation of amorphous protoplasm into sporangia containing slender tapering stalks, intricately branched capillitia, and thick-walled spores. These slime molds should be of interest to students of morphogenesis. It is important, however, to have a clear understanding of the actual sequence of events taking place during the maturation of these organisms. In a previous paper (Ross, **1957**) the development of sporangia in two genera of the Stemonitaceae was described. The following account of the development of *Lamproderma arcyrionema* Rost. is presented to add to our knowledge of differentiation in the Stemonitaceae in particular and the Myxomycetes in general.

MATERIALS AND METHODS

Developing sporangia of *L. arcyrionema* were found on a piece of rotten log obtained from the Yale Nature Preserve, New Haven, Conn., in November, 1958. The log was placed in a moist chamber and kept moist until *L. arcyrionema* arose during the afternoon of March 23, 1959. Immature sporangia were picked off the substratum with needle-nose forceps and routinely smeared in aceto-orcein at approximately 10 minute intervals. Representative stages were mounted in Hoyer's medium (Alexopoulos and Beneke, **1952**) in depression slides to retain the unsquashed appearance of the sporangia. The Hoyer mounts were particularly favorable for examining with phase contrast objectives.

OBSERVATIONS

The first indication of fruiting was the emergence of a milky-white plasmodium in patches over the surface of the log (FIG. 1). One hour



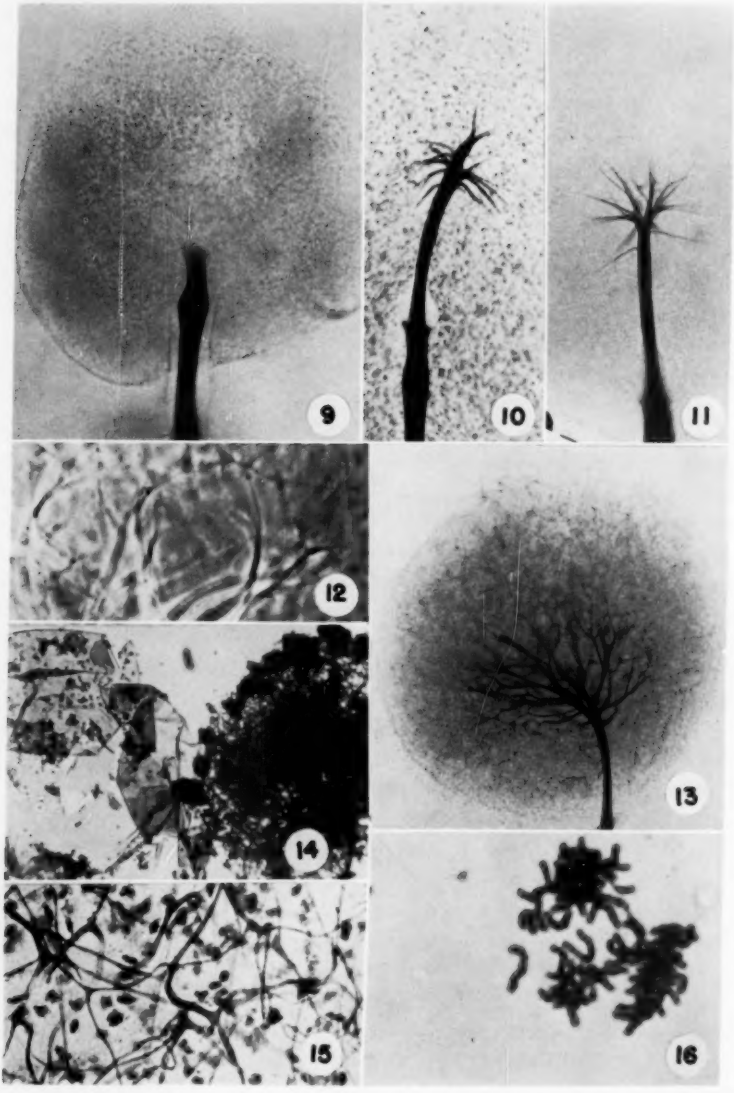
FIGS. 1–8. *Lamproderma acryrionema*. FIG. 1. First appearance of the fruiting plasmodium, hour 0, × 18. FIG. 2. Same, hour 1, × 18. FIG. 3. Same, hour 5. The stalk is partially formed and has elevated the sporangial head above the substratum, × 18. FIG. 4. Same, hour 7. The sporangium has reached its mature height, × 18.

after they had appeared the amorphous mounds gradually assumed a spherical shape and became distinct sporangial primordia (FIG. 2). For over two hours no further change was noted in the external morphology. Then the primordia were seen to be elevated above the substratum on stalks. The stalks elongated (FIG. 3) until the mature height was reached (FIG. 4) seven hours after the first sign of fruiting. The glistening sporangia remained white until the eighth hour when the color changed to a pale pink which gradually darkened to a deep red-brown in less than an hour.

During the time that the plasmodium was developing into individual sporangial primordia, no internal differentiation was seen upon examination. At the stage shown in FIG. 2, about one hour after the plasmodium had appeared on the log, a system of interlaced tubules was found under the center of each primordium (FIG. 5). The tubules were dark brown and arranged in a parallel fashion extending upward in the center, but were paler and more loosely arranged toward the outer edges of the primordium. Slides made of more mature primordia revealed that these paler and more loosely arranged tubules became darker and fused to form a sheetlike hypothallus extending under each primordium. The parallel, closely packed tubules immediately under the center of the primordium formed the base of the stalk. The stalk elongated by the gradual addition of material to the apices of these tubules, and by elongation, slowly raised the sporogenous protoplasm above the substratum (FIGS. 6, 7). Close examination of the apex of the elongating stalk revealed that the tubules were still present and distinct and had not fused into a single tube or solid structure (FIG. 8). After elongation of the stalk had been completed and just prior to formation of the capillitium, the apex of the stalk still remained as it was throughout its growth (FIG. 9). That portion of the stalk extending into the sporangium at maturity is usually termed the columella, even though there is no morphological difference.

Prior to the formation of the capillitium the membrane surrounding the sporogenous protoplasm was easily ruptured and could not be determined as a distinct structure. In FIG. 9, however, the first indication of peridium formation could be seen at the base of the sporangium as a distinct membrane. The peridium did not, at this stage, extend completely around the sporangium, although more mature sporangia showed

FIG. 5. Hypothallus and stalk initial, hour 1, × 175. FIG. 6. Elongating stalk, hour 4, × 125. FIG. 7. Elongating stalk, hour 6, × 125. FIG. 8. Apex of elongating stalk, hour 6. Individual tubules may be seen, × 750.



FIGS. 9–16. *Lamproderma arcyrionema*. FIG. 9. Sporangium mounted in Hoyer's medium at completion of stalk elongation. Peridium formation visible at base and lower left, hour 7, × 100. FIG. 10. Apical tubules bending out to form initial capillitial threads, hour 8 (aceto-orcein), × 100. FIG. 11. Bright field of sporangium mounted in Hoyer's medium (10 minutes after one in FIG. 10), × 100.

that the entire sporangium became surrounded by a peridium. Even though the peridium could now be recognized in slides, it was still very fragile and had not attained its final thickness.

Soon after the columella had reached its full height, the apical tubules separated from each other and bent out into the surrounding protoplasm to become the major capillitial threads (FIG. 10). Slides of sporangia made a few minutes later (FIG. 11) revealed little apparent change when viewed with bright field objectives, but using phase contrast objectives the capillitium was actually observed to be extended throughout the entire sporangium. The capillitium at this stage consisted of very fine tubular threads which branched and anastomosed extensively (FIG. 12). Forty-five minutes after the apical tubes had begun to bend outwards the entire capillitium was visible with bright field examination (FIG. 13), even though the individual tubes had not yet attained their mature thickness and dark coloration.

Although the peridium was first in evidence two hours before the capillitium matured, it remained as a thin, easily broken, glistening envelope and did not become a firm structure until after spore formation. After cleavage of protoplasm and the formation of spore walls the peridium dried out and became a definite irridescent structure which could be removed in its entirety from the sporangium (FIG. 14).

The nuclei in the sporangium were in the interphase condition during the development of the stalk and capillitium. When the capillitium was almost mature but before attaining its final thickness and coloration, the nuclei entered prophase of Meiosis I (FIG. 15). The divisions proceeded very rapidly, and slides made thirty minutes after the first prophases were noted contained only telophase nuclei and cleavage patterns, indicating that the meiotic divisions had been completed. The chromosome number was found to be 53 ± 2 at Metaphase II (FIG. 16).

DISCUSSION

In common with other members of the Stemonitaceae, *L. arcyrionema* produces an internal stalk and a hypothallus underneath the sporangial primordia and not on the upper surface of the fruiting plasmodium as is found in the Physarales and Trichiales (Ross, **1957**, **1959**). The

FIG. 12. Phase contrast view of capillitium of sporangium in FIG. 11, × 500. FIG. 13. Fully extended capillitium before final thickening of threads (Hoyer's), hour 9, × 75. FIG. 14. Mature sporangium (right) and peridium (left), × 75. FIG. 15. Capillitial threads and prophase of Meiosis I, hour 9. The hollow nature of the tubes can be seen, × 400. FIG. 16. Chromosomes at Metaphase II, × 2000.

development of *L. arcyrionema* follows closely the pattern described for *Comatricha typhoides* (Bull.) Rost. (Ross, **1957**). The major difference is that in *Comatricha* the tubules composing the columella bend out at intervals along the whole length of the columella, rather than bending out only at the apex as in *Lamproderma*. This difference is responsible for the two distinct types of capillitia used in the taxonomic separation of the two genera.

Since the development of all the sporangia produced by the plasmodium observed in this investigation matured simultaneously, it was possible to follow the sequence of sporangial development by preparing slides at intervals. The development of the capillitium was indeed striking. In FIG. 10 the capillitium has not formed apart from the initial apical tubules, yet 10 minutes later, in FIG. 12, the capillitium extends throughout the sporangium. It was not observed whether the development of the capillitium proceeds from the apical tubules outwards, or whether it is laid down all at once on a preformed system of tubes or vacuoles in the cytoplasm which then connects up with the apical threads. The initial capillitial threads are the apices of the stalk tubules which bend out into the protoplasm and have been formed by the gradual addition of material to their ends. If this process of further addition of material to the ends of the tubules is the means by which the capillitium extends through the sporangium, there must be a considerable speeding up of the process to complete capillitium extension in so short a time. The final thickening of the capillitium requires a longer time and can be correlated to the color changes noted in the external appearance of the sporangia. When the capillitium is mature, but before the spore walls have formed, the sporangia are a glistening deep red-brown color.

The peridium of *Lamproderma* is recognized as being a much firmer and more persistent structure than that of *Comatricha* or *Stemonitis*. But, in common with those genera, the peridium of *L. arcyrionema* does not form until late in the development of the sporangium and does not attain its final thickness and rigidity until after all other morphogenetical processes are complete. Although the initial thickening of the membrane begins while the sporogenous protoplasm is still undifferentiated, the final stage of the firm, removable peridium is completed after all protoplasm has been encased in spore walls and is presumably no longer actively controlling differentiation.

The reduction divisions in *L. arcyrionema* occur at the same time as those of the other members of the Myxogastres described by Wilson and Ross (**1955**); that is, just prior to cleavage and after the capillitium has

differentiated. The rapidity of the meiotic divisions and the relatively high chromosome number are also typical of most of the Myxomycetes studied previously by the writer.

SUMMARY

The development of *Lamproderma arcyrionema* is traced from the first emergence of the fruiting plasmodium on the surface of a rotten log to the final maturation of the sporangia. The formation of the stalk and capillitium is described and found to follow the pattern typical of members of the Stemonitaceae. The chromosome number is reported to be $N = 53 \pm 2$.

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PHYSIOLOGY OF WOOD-ROTTING BASIDIOMYCETES. III. STUDIES ON THE UTILIZATION OF OPTICAL ISOMERS OF AMINO ACIDS[1]

MARSHALL W. JENNISON AND ALEXANDER M. PERRITT[2]

It is clear that all amino acids, even on an equivalent nitrogen basis, are not of equal value in fungus nutrition, either for fungi in general (**5**) or for wood-rotting Basidiomycetes in particular (**7**). Both chemical structure, e.g., length of the carbon chain or position of the amino group (**6**, **7**, **11**, **12**, **19**, **20**), and isomeric configuration are the main intrinsic factors determining assimilation of amino acids. Generally, the L- (natural) isomer is better or exclusively utilized; the D- (unnatural) isomer may actually be inhibitory (**4**, **10**, **23**). For particular fungi and amino acids, utilization of D-isomers is specific. Although a number of bacteria have been investigated for their ability to utilize D-amino acids, not many yeasts or filamentous fungi have been examined in this regard (**17**). Amino acid oxidases of but few filamentous fungi have been studied (**3**, **6**, **9**, **14**, **18**, **21**, **22**); the role of D-amino acid oxidases and the probable mechanisms of the utilization of D-amino acids by fungi have been discussed by a few workers (**4**, **5**, **6**, **10**, **13**, **17**, **18**).

Only three comparative studies have come to our attention on the action of Basidiomycetes on different optical isomers of amino acids. *Ustilago scabiosae* assimilated both L- and D-isomers of certain amino acids, but only the L- form of others (**2**); extracts of *Psalliota campestris* were shown to hydrolyze both optical isomers of the amides of proline, alanine and leucine (**16**); and we have previously reported (**7**), but without presenting protocols, that 10 species representing 8 genera of wood-rotting Basidiomycetes were able to utilize only the L-isomer of tryptophane and of leucine. Also, in the latter study, using a series

[1] These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Syracuse University (N-onr-669(06)), under the direction of M. W. Jennison. This report constitutes a technical report under the above contract. Reproduction in whole or in part is permitted for any purpose of the United States Government.

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of concentrations of L-isomers and of racemates, it was found that within limits the amount of growth was directly proportional to the nitrogen concentration of the L-isomer.

The present investigation is a quantitative study of the utilization—by 6 species of wood-rotting fungi—of alanine and of aspartic acid, using racemic mixtures and D-isomers as well as the L- forms. These two amino acids are comparable on the basis of simple chemical structure and short chain length.

MATERIALS AND METHODS

The cultures were obtained from the U. S. Department of Agriculture, Beltsville, Maryland, and were maintained on slants of potato dextrose agar (Difco, dehydrated). The amino acid isomers and racemic mixtures (Nutritional Biochemicals Corp.) were, except for L-aspartic acid, synthetic and chromatographically pure.

TABLE I

BASAL SYNTHETIC MEDIUM

D-Glucose	10.0 g
KH_2PO_4	1.5 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Thiamine hydrochloride	1.0 mg
Amino acid	To give 30 or 60 mg *nitrogen* (see text)

Trace elements: B, 0.10 mg; Cu, 0.01 mg; Fe, 0.05 mg; Mn, 0.01 mg; Mo, 0.01 mg; Zn, 0.07 mg

Distilled water: To make 1 liter

All growth studies were carried out in shake culture, using 70 ml of medium in 250-ml Erlenmeyer flasks and incubating at 28° C. In place of cotton plugs, which may introduce traces of extraneous nutrients into media, flasks were capped with a 4-inch square of special cellophane (Dupont, type 450 PT) which withstands autoclaving, held in place loosely by a rubber band. To obtain reproducible quantitative results, procedures were carefully controlled and standardized.

To determine amino acid utilization, a chemically-defined basal medium was used, the composition of which is given in TABLE I.

To this basal medium each L- or D-amino acid was added singly to give a concentration of 0.003 per cent (30 mg/liter) *total nitrogen;* racemic mixtures (DL-) were added in concentrations of both 0.003 and

0.006 per cent total nitrogen, on the assumption that only the natural (L-) forms of the acids were utilizable and that the amount of growth would be proportional to the "utilizable" (L-) nitrogen concentration. The different forms of each test amino acid should therefore be comparable on an equivalent "utilizable" nitrogen basis. Media were adjusted to give a pH of 5.0–5.5 after sterilization. In preparing the basal medium the inorganic salts, glucose, amino acid and thiamine were sterilized separately at 121° C for 20 minutes, then combined aseptically.

All quantitative data on growth in the amino acids are based on the average dry weight of mycelium produced in the test media in duplicate flasks. The contents of each flask were filtered on an alundum crucible, the mycelial pellets washed, then dried to constant weight at 80° C in a vacuum oven.

To produce the inoculum for determining utilization of the different forms of a given amino acid, mycelium from a stock culture was transferred to the basal medium containing the L-isomer of the acid and grown for 7 days in shake culture. The resulting growth of mycelial pellets was blended for one minute in a Waring blendor, washed three times in distilled water by centrifuging at 2000 rev/min for two minutes, and resuspended in 35 ml of distilled water. The test media and controls for each organism were inoculated with this suspension, using 0.2 ml (equivalent to about 0.15 mg oven-dry weight of mycelium) per flask, and incubated for 7–22 days, depending upon the organism. Control flasks (without nitrogen) showed virtually no growth (TABLE II), indicating little carry-over of L-amino acid by the primary inoculum. There was, therefore, no need for serial subculture in the test media to insure that growth was due to the particular form of amino acid under test.

RESULTS AND DISCUSSION

TABLE II shows the results obtained with the several species of wood-rotting fungi in the indicated concentrations and forms of amino acids in the basal medium. All data are averages of duplicate flasks.

It is seen that there was little or no growth in the synthetic medium in the absence of added nitrogen, showing that the carry-over of L-amino acids by the original inoculum was nil. The data also show that the amounts of growth in the D-isomers alone were in all cases approximately the same as in the control media, and therefore do not indicate assimilation of the unnatural forms alone under the experimental conditions used. Generally, the amounts of growth in the racemates at 0.003 per cent *total* nitrogen concentration (0.0015 per cent "utilizable" nitrogen) were ap-

proximately one-half as large as in the respective L-isomers at 0.003 per cent nitrogen, as would be expected if the D-forms were not assimilated and the amounts of growth were directly proportional to the "utilizable" (L-) nitrogen concentration. The outstanding exception was *Lenzites trabea* in alanine, where the amount of growth in the racemic mixture at 0.003 per cent *total* nitrogen was the same as the mycelial weight in the L-isomer (0.003 per cent "utilizable" nitrogen). This suggests assimilation of the D-isomer of the racemate in the presence of the L- form,

TABLE II

GROWTH OF WOOD-ROTTING FUNGI IN L-, D-, AND DL-FORMS OF AMINO ACIDS IN BASAL SYNTHETIC MEDIUM. DRY MYCELIAL WEIGHT PER 70 ML CULTURE AFTER INDICATED PERIODS OF INCUBATION IN SHAKE CULTURE AT 28° C

Amino acid in basal medium	Amino acid form	Amino acid nitrogen in medium, per cent	Organism and original culture number					
			Polyporus tulipiferus Mad. 517	*Polyporus palustris* FP 94152	*Lenzites trabea* Mad. 539	*Fomes subroseus* Snell 20	*Peniophora gigantea* FP 56475-S	*Lentinus lepideus* Mad. 534
			Age of culture, days					
			7	7	14	21	15	22
			Amount of growth, mg					
None	None	0	0	2.0	1.3	3.7	2.7	0.9
Alanine	L-	0.003	74.0	43.3	20.6	38.6	51.2	43.8
	D-	0.003	5.0	2.6	3.0	2.0	1.6	0
	DL-	0.003	43.9	14.9	21.7	17.2	21.7	23.6
	DL-	0.006	75.6	37.9	19.2	41.0	28.4	32.6
Aspartic acid	L-	0.003	65.3	37.1	34.9	33.8	42.2	42.4
	D-	0.003	5.5	4.5	3.0	3.7	1.3	1.8
	DL-	0.003	45.0	21.5	24.1	17.0	27.0	22.4
	DL-	0.006	61.3	33.1	36.9	31.5	43.0	45.8

although there was no more growth in the racemate at 0.006 per cent nitrogen than at 0.003 per cent, and growth in the unnatural form alone was nil. In certain other cases—e.g., *Polyporus tulipiferus, L. trabea* and *Peniophora gigantea* in aspartic acid, and *Polyporus palustris* in alanine—there were smaller disparities between the amounts of growth "expected" in the racemates relative to the amounts in the L-amino acids (1:1), with both forms at 0.003 per cent total nitrogen concentration. Whether these disparities were within the experimental error, were a consequence of the lack of proportionate growth at different L-isomer

nitrogen concentrations, or resulted from the partial utilization of—or, with *P. palustris*, inhibition by—the D-isomer of the racemate, is not certain.

Growth in the racemic mixtures at 0.006 per cent total nitrogen was in most cases virtually the same as the amounts of growth in the respective L-isomers at 0.003 per cent nitrogen (exceptions: *P. gigantea* in alanine and to a lesser extent *Lentinus lepideus* in alanine), and approximated—with the outstanding exception of *L. trabea* in alanine—double the growth in the racemates at 0.003 per cent total nitrogen. These generally consistent results are what might be expected on an equivalent utilizable nitrogen basis. The reasons for the seeming inconsistencies in the amounts of growth with *P. gigantea* and *L. lepideus* are not clear, although inhibition of growth by the D-isomer of the racemate is a possibility. With *L. trabea*, the disparate ratio in mycelial weights (1:1 instead of 2:1) in the two concentrations of the racemate of alanine may have resulted from utilization of the D-isomer of the racemate at the 0.003 per cent total nitrogen concentration, as mentioned above.

The above results for alanine and aspartic acid are similar—except for *L. trabea* in alanine—to our previous findings for tryptophane and leucine (**7**), in that the wood-rotting fungi previously investigated—including *P. tulipiferus*, *P. palustris*, *L. trabea* and *P. gigantea*, which were also used in the present experiments—utilized the L-isomer but not the D- form of the amino acids. The results show clearly that isomerism plays an important part in the utilization of amino acids by wood-rotting fungi.

On the equivalent "utilizable" nitrogen basis used in these experiments, the two structurally simple amino acids gave similar quantitative results with a given organism. The differences in amounts of growth among organisms in a given nitrogen source were due in part, at least, to different incubation periods of the several species and in part to different environmental requirements for maximum growth of the organisms, e.g., pH, oxidation-reduction potential and temperature (**7**). It has also been shown with strains of a nitrogen-fixing bacterium that the maximum growth attained on any one amino acid isomer was a function of pH and temperature (**8**).

Our generally consistent findings that the racemates of the amino acids supported about one-half as much growth as the L-isomers, on an equal *weight* basis, are in accord with results of others (**15**) on lactic acid bacteria; with these bacteria, the DL- form of tryptophane was about 50 per cent as active as the L-isomer.

Whether, in the exceptions to the "expected" growth ratios cited above, certain of the organisms (*L. trabea* in alanine and in aspartic acid, and *P. tulipiferus* and *P. gigantea* in aspartic acid) actually utilized the D-isomers of the racemates in the presence of the L-isomers, or, in other cases (*P. gigantea* and *L. lepideus* in alanine) were inhibited by the D- form, cannot be determined unequivocally from the data. Although for bacteria and higher fungi the unnatural form has often been found to be inhibitory (**4**, **10**, **15**, **23**), it is possible that in rats, at least, the utilization of a D-isomer may be enhanced by the presence in the diet of traces of the L- form (**1**). Further, the utilization of D-amino acids depends principally on the rate of conversion to the L-isomer, and the presence of D-amino acid oxidase is a necessary, although not always sufficient factor for the utilization of the D-form (**13**); the time factor and other conditions must also be important. Specific racemases effecting direct interconversion of the D- and L- forms are known in bacteria but not in higher fungi.

SUMMARY

The growth of the wood-rotting fungi, *Polyporus tulipiferus, P. palustris, Lenzites trabea, Fomes subroseus, Peniophora gigantea* and *Lentinus lepideus,* was determined in L-, D- and DL-alanine and in L-, D- and DL-aspartic acid, separately, in shake culture in a basal synthetic medium. Generally, the amounts of growth in the racemic mixtures at 0.003 per cent *total* nitrogen concentration approximated one-half of that in the L-isomers at the same total nitrogen concentration, as would be expected if the D- forms were not utilized and the amounts of growth were directly proportional to the "utilizable" (L-) nitrogen concentrations. Generally, also, mycelial weights in the racemic mixtures at 0.006 per cent total nitrogen were the same as the amounts of growth in the respective L-isomers at 0.003 per cent nitrogen, and approximated double the growth in the racemates at 0.003 per cent nitrogen. In no case was there utilization of D-isomers alone, and in only one case —*L. trabea* in alanine—was there some evidence for the assimilation of the D-isomer of the racemate in the presence of the L- form. In one or two cases there was a suggestion of some inhibition of growth by the D-form in the racemic mixture. The isomeric configuration of amino acids is an important factor in their utilization by wood-rotting fungi.

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FUNGI ISOLATED IN CULTURE FROM SOILS OF THE NEVADA TEST SITE [1]

L. W. Durrell [2] and Lora Mangum Shields [3]

(with 10 figures)

Introduction.—Of the numerous investigations of soil fungi, none has focused upon the terrestrial fungal flora of the semidesert southwest. Though seasonally dry, this area is subject to periods of precipitation which provide moisture adequate for plant growth. Further, soil fungi, like algae, are known to survive long intervals of extreme heat and drought.

Five hundred and fifty soil samples from the Nevada Test Site were cultured on media selective for fungi. The fungal flora of the arid soils from neighboring Death Valley was inventoried in culture for comparison. A limited number of the species isolated were irradiated with ultraviolet light to evaluate relative resistance to this wave length present in greater amounts in desert environments.

Methods.—Samples obtained with a sterilized trowel from the surface and from the 3- to 6-inch depth were sealed directly into freezer bags at the collection site. Fungi were isolated by the soil plate technique, a method not subject to the disadvantage of favoring profuse spore formers. Approximately one-half gram of each sample was transferred with a sterile spatula to a Petri dish and covered with Rose-Bengal agar at 45° C. On this substrate, with streptomycin as a component, bacterial growth is inhibited, and the fungi form discrete colonies. All cultures were maintained at room temperature. Plates were examined microscopically at intervals for one to several weeks until no additional species were encountered. The presence or absence of a species on each plate was recorded, but relative abundance was not quantitated.

The samples from the Nevada Test Site were collected during October, December and March within the two internal drainage basins,

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[2] Dean Emeritus, Colorado State University, Collaborator.

[3] Project director; Chairman, Department of Biology, New Mexico Highlands University.

Frenchman Flat and Yucca Flat, at altitudinal ranges between 3100 and 4500 feet. The seed plant cover of this area grades from a predominantly southern desert type of vegetation at lower altitudes through transitional species and into northern desert shrub appearing at around 4500 feet. Annual precipitation averages about four inches, of which approximately one-third falls as snow. Soil types range from the

TABLE I

FUNGI ISOLATED ON ROSE-BENGAL AGAR (CONTAINING STREPTOMYCIN) FROM 550 SOIL SAMPLES FROM THE NEVADA TEST SITE

A. Fungi producing darkly pigmented spores, or mycelium, or both.

1. **Alternaria tenuis* Nees
2. *Aspergillus wentii* Wehmer
3. *Cladosporium herbarum* (Persoon) Link
4. **Phoma* sp.
5. *Pullularia pullulans* (deBary) Berkhout
6. *Rhizopus nigricans* Ehrenberg
7. **Stachybotrys atra* Corda
8. **Stemphylium ilicis* Tengwall
9. *Stysanus medius* Sacc.
10. **Tetracoccosporium paxianum* Szabo

B. Fungi producing unpigmented or light colored spores, or mycelium, or both.

11. *Aspergillus flavipes* Bainier & Sartory
12. *A. fumigatus* Fresenius
13. *A. niveum* Blochwitz
14. *A. ochraceus* Wilhelm
15. *A. restrictus* G. Smith
16. *A. sulfureus* (Fres.) Thom & Church
17. *A. ustus* (Bainier) Thom & Church
18. *A. versicolor* (Vuillemin) Tiraboschi
19. *Botrytis bassiana* Balsamo
20. *Cephalosporium* sp.
21. *Chaetomium aureum* Chivers
22. *C. spirale* Zopf
23. *Choanephora* sp.
24. **Circinella muscae* (Sorokine) Berlese & deToni
25. *Coccosporium* sp.
26. *Cunninghamella bainieri* Naumov
27. *C. microspora* (Riv.) Matr.
28. *Fusarium roseum* Link emend. Snyder & Hansen
29. *Gliocladium penicilloides* Corda
30. *G. roseum* (Link) Thom
31. *Mucor corticolus* Hagem
32. **Mucor spinescens* Lendner
33. *M. varians* Povah
34. *Myrothecium verrucaria* (Albertini & Schweinitz) Ditmar
35. *Penicillium granulatum* Bainier
36. **P. oxalicum* Thom
37. *P. urtica* Bainier
38. **Pythium mamillatum* Meurs
39. *Streptomyces* sp.
40. *Syncephalastrum racemosum* (Cohn) Schroeter
41. *Trichoderma viride* Corda

* Species in TABLE I which are marked by an asterisk are illustrated, FIGS. 1-9.

highly impervious silt adjacent to the playa in each basin to coarse-textured alluvial fill toward the valley margins. Within the test site, soils were collected in the undisturbed plant cover and within the 1.0 mile radius of ground zero sites of nuclear tests two years previously.

Observations.—Fungal growth, profuse in most cultures, developed from all soils plated except from certain samples exposed in laboratory to ultraviolet light for different periods. A wider distribution and increased incidence of fungi appeared to be related to factors favoring the accumulation and retention of soil moisture. The qualitative distribution of species in the vicinity of ground zero sites was not different from that in the undisturbed plant cover. Similarly, the surface fungal flora differed only quantitatively from that at the 3- to 6-inch depth. *Stemphylium ilicis,* as an example, developed from 78 per cent of the surface and 60 per cent of the subsurface cultures in the first 170 platings. All of the species isolated were common soil fungi. Four taxa developed

TABLE II

SPECIES OF FUNGI ISOLATED OF ROSE-BENGAL AGAR FROM SOIL SAMPLES FROM DEATH VALLEY

1. *Alternaria tenuis* Nees
2. *Aspergillus candidus* Link
3. *A. niger* van Tieghem
4. *A. wentii* Wehmer
5. *Camarosporium* sp.
6. *Coniothyrium* sp.
7. *Fusarium* sp.
8. *Hormodendrum nigrescens* Paine
9. *Macrophomina phaseoli* (Maublanc) Ashby
10. *Penicillium oxalicum* Thom
11. *Pullularia pullulans* (de Bary) Berkhout
12. *Rhizopus nigricans* Ehrenberg
13. *Stemphylium botryosum* Wallr.
14. *S. ilicis* Tengwall

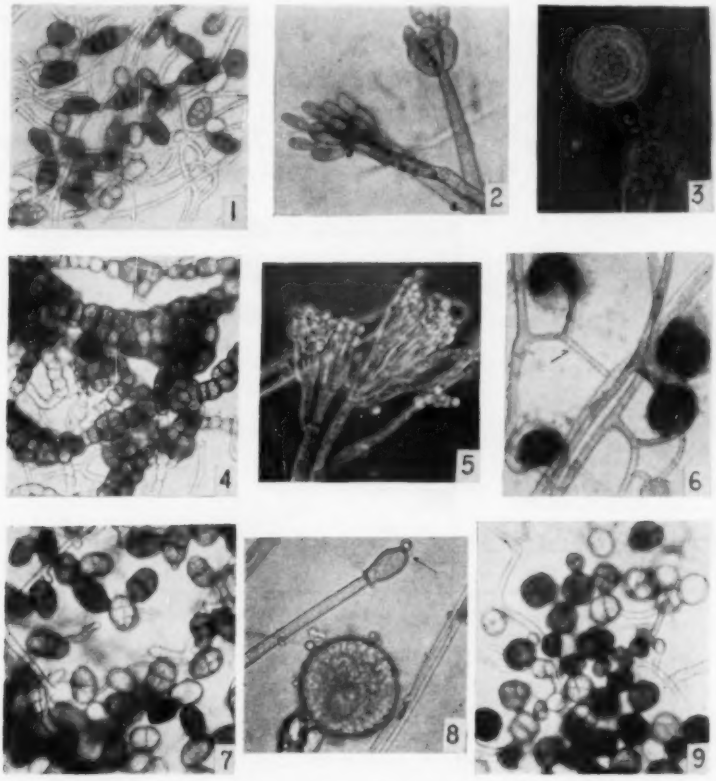
in from 40 to over 80 per cent of the samples cultured in different series: *Stemphylium ilicis, Fusarium* sp., *Phoma* sp. and *Penicillium oxalicum* (listed in order of decreasing frequency of appearance). A number of species developed in only an occasional sample. *Stachybotrys atra,* as an example, rarely appeared except on impervious playa-type soil from the lowest part of the internal drainage basins.

Ten of the 41 fungal species isolated from soils of the Nevada Test Site produce dark spores, or mycelium, or both (TABLE I). On many plates the dark-spored species predominated to the extent that the entire growth assumed a sooty aspect.

A still higher percentage of dark-spored species developed from soils collected in Death Valley (TABLE II). Of the seven taxa from Death Valley common also to the Nevada Test Site (1, 4, 7, 10, 11, 12 and 14), five produce dark spores. One set of 50 plates, cultured from Death Valley soils, was predominantly black from growth of *Stemphylium* sp.,

with occasionally one or two colonies of *Penicillium oxalicum* or *Fusarium* sp. per plate.

The predominance of black-spored species in soils from the Nevada Test Site as well as from Death Valley suggests that in strongly insolated



FIGS. 1–9. Nine of the 41 taxa of fungi isolated in culture from Nevada soils. FIG. 1. *Alternaria tenuis*. FIG. 2. *Stachybotrys atra*. FIG. 3. *Pythium mamillatum*. FIG. 4. *Phoma* sp. FIG. 5. *Penicillium oxalicum*. FIG. 6. *Circinella muscae*. FIG. 7. *Stemphylium ilicis*. FIG. 8. *Mucor spinescens*. FIG. 9. *Tetracoccosporium paxianum*.

regions the melanin pigment affords a degree of protection. To determine relative resistance of fungal spores to ultraviolet light, cultures of a number of species were exposed to wave length 2573 Å for different periods at 40 $\mu w/cm^2$ per minute. The time interval within which this exposure was 100 per cent lethal is shown by graph for seven repre-

sentative species from the Nevada Test Site of a total of 36 tested (FIG. 10). Certain dark-spored species, as *Stemphylium ilicis, Stachybotrys atra* and *Cladosporium herbarum,* survive prolonged exposures. *Stemphylium ilicis* spores are particularly resistant, germinating after 60 minutes in 95 to 100 per cent of the exposed samples. Spores of *Aspergillus niger,* also black, were killed by two minutes exposure to ultraviolet light of the wave length 2573 Å (FIG. 10).

The usual organic solvents do not extract the pigment from *Stemphylium ilicis* spores, while the spore pigment of *Aspergillus niger* was found to dissolve readily in methyl alcohol. Thin layers of these spores attached to a quartz slide were checked by a spectrograph and electro-

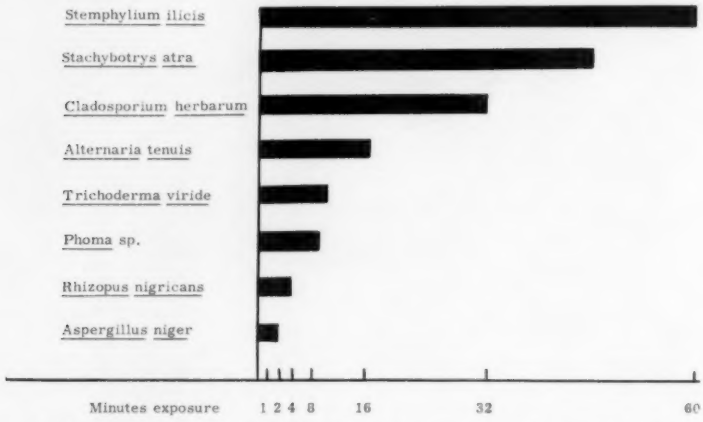


FIG. 10. Graph showing survival time of fungus spores following exposure to ultraviolet light.

spectrometer for determining light transmission. No light within the range of 2000 Å to 20,000 Å passed through *Stemphylium* spores. In contrast, the extracted pigment from *Aspergillus niger* spores showed a wide transmission band with a peak maximum at 12,000 Å. Spore or nuclear volume also may modify the effect of ultraviolet light. Possibly energy absorption is less rapid by large spores, as in *Stemphylium* sp., than by the small spores of *Aspergillus niger* with about 1/50 the volume. The dark spores of *Coccosporium,* however, with an approximate volume of 13,000 μ^3 were killed in half the time required for *Stemphylium* with a spore volume of 8000 μ^3.

Summary.—In culture on Rose-Bengal agar containing streptomycin, 41 fungal taxa were isolated from surface and 3- to 6-inch samples of

arid soils of the Nevada Test Site. Four taxa developed in from 40 to over 80 per cent of the samples cultured: *Stemphylium ilicis, Fusarium* sp., *Phoma* sp. and *Penicillium oxalicum*. Certain of the species which produced black spores tended to predominate in culture. Five of these species were also among the 14 isolated from Death Valley soils. The black spores of *Stemphylium ilicis* were found to survive 60-minute exposures to ultraviolet light of the wave length 2573 Å. The melanin pigment may serve a protective function in highly insolated desert regions, although not all species producing dark spores are especially resistant to ultraviolet light.

THE INFLUENCE OF TEMPERATURE AND RELATIVE HUMIDITY ON GROWTH AND SURVIVAL OF SILAGE FUNGI [1]

ROBERT D. BONNER AND CHARLES L. FERGUS [2]

INTRODUCTION

Temperature and relative humidity are environmental factors important in determining whether fungi can subsist or grow on vegetable substrates. Their effects have been studied largely in connection with the prevalence of fungi on substrates in storage. Chistiakov (**3**) found that most fungi may be grouped into three categories on a thermal basis, *viz.*, psychrophiles, mesophiles and thermophiles. Fungi associated with feeds are in all three groups.

Snow (**5**) demonstrated that a large number of fungi found associated with feeds, especially cereal and grain, were able to develop at relative humidities of 90 to 100 per cent. The number of fungi that could develop on vegetable substrates below a moisture content equivalent to 90 per cent relative humidity was found to be considerably limited, although viable inoculum could be demonstrated to be present by cultural studies. In general it has been shown that members of the Mucoraceae and Deuteromycetes will grow on substrates stored at relative humidities of 90–100 per cent and not below; however, some species of *Penicillium* will grow at humidities as low as 75 per cent. Members of the *Aspergillus glaucus* group have even been found growing in humidities as low as 65 per cent relative humidity.

Semeniuk (**4**) states that adjustment of moisture content of feeds equivalent to 65 per cent relative humidity is borderline for a long time storage, while higher moisture contents of feeds, equivalent to 70 per cent relative humidity, may be allowed for short time storage at low tempera-

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tures. Semeniuk further points out that low temperatures have been effective only in reducing or controlling the activity of most fungi, since they easily survive very low temperatures. However, they do not survive higher temperatures, especially those above their maxima, for any length of time.

The effects of temperature and relative humidity on the growth and survival of ten selected fungus isolates from silages were studied.

MATERIALS AND METHODS

Battery jars containing chemical solutions (**1**, **6**, **7**) were used to yield six relative humidities at 25°, 37°, and 50° C (TABLE I).

TABLE I

PER CENT RELATIVE HUMIDITY AT THREE TEMPERATURES OVER VARIOUS CHEMICAL SOLUTIONS

Chemical	23.5–25° C	37° C	50° C
H_2O	100	100	100
$NH_4H_2PO_4$	93	91	—
NaCl (120 g/liter)	—	—	92.6
K_2CrO_4	86.5	85.6	—
NaCl (240 g/liter)	—	—	85
$(NH_4)_2SO_4$	80	79	—
NaCl (320 g/liter)	75.5	75	79
H_2SO_4 (31.4%)	—	—	75
H_2SO_4 (37.1%)	—	—	65
$NaC_2H_3O_2 \cdot 3H_2O$	—	67.7	—
$NaNO_2$	64.8	—	—

The fungi investigated were: *Alternaria tenuis* Nees ex Fr., *Aspergillus fumigatus* Fres., *Chlamydomyces palmarum* (Cke.) Mason, *Cladosporium clatum* (Harz.) Nannf., *Fusarium moniliforme* Sheldon, *Monascus purpureus* Went, *Mucor janssenii* Lend., *Mucor racemosus* Fres., *Geotrichum candidum* Link ex Pers., and *Penicillium purpurogenum* Stoll. All were isolated from grass ensilage, except *Cladosporium clatum* which was isolated from alfalfa ensilage.

Ten fungi were selected because they were components of the flora of "suspected" moldy silages (**2**); they represent six different fungus families; they exhibit different growth habits; and the influence of temperature and humidity on their growth and survival had not been previously studied.

A soybean ensilage having an original moisture content of 56 per cent (oven-dry weight basis) was used as the substrate.

Five-tenths ml suspensions of spores and mycelial fragments were used to inoculate one gram samples of the ensilage in 50 ml Erlenmeyer flasks. Cultures were placed at the established humidities and temperatures 18 hours following inoculation, to allow spores to germinate prior to exposure to the different temperatures and relative humidities. Periodic visual examinations of cultures were made in order to record the rate and extent of growth over a period of 30 days. The growth of each fungus was compared to the growth on malt extract agar at 25° C. The maximum observed growth, comparable to growth on malt extract, was designated "5" and the least "trace." Inoculated substrates that ex-

TABLE II

THE EFFECT OF RELATIVE HUMIDITY AND TEMPERATURE ON GROWTH[a] OF 10 FUNGI ON SOYBEAN ENSILAGE

Fungus	% Relative humidity											
	100		92		86		80		75		65	
	° C											
	25	37	25	37	25	37	25	37	25	37	25	37
Alternaria tenuis	5	5	4	5	4	4	4	T	3	T	0	0
Aspergillus fumigatus	5	5	5	5	5	5	5	3	4	3	2	1
Chlamydomyces palmarum	5	3	5	3	2	2	2	0	0	0	0	0
Cladosporium elatum	5	4	4	4	4	4	4	T	3	0	0	0
Fusarium moniliforme	5	5	5	5	4	5	4	1	3	T	T	0
Monascus purpureus	5	5	5	5	5	4	5	2	4	1	2	1
Mucor jansseni	5	3	5	3	5	2	5	0	2	0	0	0
Mucor racemosus	5	2	5	2	5	0	5	0	3	0	0	0
Geotrichum candidum	5	3	3	3	2	2	2	0	0	0	0	0
Penicillium purpurogenum	5	5	5	5	5	5	5	0	4	0	0	0

[a] Based upon visual observations of cultures after 30 days. The 5 is maximum growth and is equal to that made on malt extract agar. T is trace.

hibited no visually detectable growth after 30 days were tested to determine their viability, i.e., survival, by placing them on malt extract agar and incubating at 25° C.

RESULTS

The ten fungi exhibited considerable differences in growth over a 30-day period at the three temperatures and six relative humidities. All of the fungi developed at 25° and 37° C in atmospheres above 85 per cent relative humidity (TABLE II). No growth was observed at 50° C. Considerable differences in amount of growth of the various fungi were

easily detected by visual estimation. The maximum growth occurred at 25° C and 100 per cent relative humidity for most of the species, and a gradual reduction was noted as the relative humidities were lowered. A very sharp decline was noted below 80 per cent relative humidity. The amount of growth produced at 25° by most species was greater than that at 37° and comparable relative humidities.

The inocula of flasks that showed no visible growth at 50° and at certain relative humidities at 25° and 37° were tested for viability. All of those at 25° and 37° showed excellent growth when transferred to malt extract agar. At 50° the inoculum of *Aspergillus fumigatus* and *Monascus purpureus* survived exposure at relative humidities of 100 and 92 per cent. *Penicillium purpurogenum* survived at relative humidities of 92 and 86, but not at 100 per cent. None of the other fungi survived the 30 days exposure.

The per cent moisture content of the ensilage changed over the 30-day period at the different relative humidities. The ensilage had an original moisture content of 56 per cent. At 100 per cent relative humidity the moisture content changed to 90. At 65 per cent relative humidity the ensilage changed to 20 per cent moisture content.

DISCUSSION

A considerable number of studies have been made on the ecology of molds. The factors found to be most important, aside from nutrition, have been temperature, relative humidity, moisture content of the substratum and oxygen relations. In this investigation, temperature and relative humidity, and indirectly moisture content of the substratum, were also found to be important. The species selected were found to belong to at least four growth types: (a) species that developed very rapidly and produced large numbers of spores in favorable environmental conditions, represented by *Fusarium moniliforme, Mucor janssenii,* and *Mucor racemosus,* typical so-called "molds"; (b) species that developed rapidly over a wide range of conditions producing moderate amounts of mycelium and spores, represented by *Aspergillus fumigatus, Monascus purpureus,* and *Penicillium purpurogenum;* (c) species that produced thick mycelial colonies under favorable conditions and very thin growth under adverse conditions, represented by *Alternaria tenuis, Chlamydomyces palmarum,* and *Cladosporium elatum;* and (d) species that were highly restricted under all conditions studied, represented by *Geotrichum candidum.*

The information obtained in this study further substantiates previous

work as to the importance of these environmental factors in the molding of silages and feeds. Semeniuk's recommendation (**4**) that adjustment of moisture content of feeds equivalent to 65 per cent relative humidity is borderline for long time storage is also applicable to silage. Only *Aspergillus fumigatus* and *Monascus purpureus* grew at this humidity level, and growth was sparse.

The ability of spores of *Aspergillus fumigatus, Monascus purpureus* and *Penicillium purpurogenum* to survive exposure to 50° C for 30 days is remarkable. However, they survived only under conditions of high relative humidity.

SUMMARY

The influence of 3 temperatures and 6 relative humidities upon the growth and survival of ten fungi isolated from moldy silages was studied. The fungi were *Alternaria tenuis, Aspergillus fumigatus, Chlamydomyces palmarum, Cladosporium elatum, Fusarium moniliforme, Monascus purpureus, Mucor janssenii, Mucor racemosus, Geotrichum candidum,* and *Penicillium purpurogenum.* No growth was observed at 50° C, but all developed at 25° and 37° in atmospheres above 85 per cent relative humidity on soybean ensilage. Maximum growth occurred at 25° and 100 per cent relative humidity, with a gradual reduction as the relative humidities were lowered. A sharp decline was noted below 80 per cent relative humidity. *Aspergillus fumigatus* and *Monascus purpureus* were the only fungi able to grow at 65 per cent relative humidity. The inoculum of *A. fumigatus, M. purpureus,* and *Penicillium purpurogenum* survived 30 days exposure to 50° at high humidities. None of the other fungi survived the exposure.

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NOMENCLATURAL NOTES. IV. THE GENERIC NAME PLICARIA

RICHARD P. KORF

The genus *Plicaria* was published by Fuckel (**1870**) and originally contained descriptions of four spherical-spored and eight oval-spored species of discomycetes, without indication of a generic type. In the succeeding 90 years the name has been used with different interpretations at the generic and at infrageneric ranks. In most of the literature written in French the name has been used for spherical-spored species in which the asci blue with iodine, in the German literature mostly for oval-spored species with similarly iodine-positive asci, while in the books and papers written in English the name has for the most part been relegated to synonymy.

The application of the name *Plicaria* depends upon the selection of a lectotype from among the original species included in the genus. I have investigated the problem 1) at the request of my former student, Dr. L. R. Batra, who desired to know the availability of certain generic names for spherical-spored species from India (Batra, **1961**), and 2) because of the publication of a name by Dr. Roswitha Schneider (**1954**) for an intriguing discomycete with an *Ostracoderma* imperfect state with which I have been working at intervals since 1947.

The earliest attempt at typification which I have located was an indication by Saccardo (**1884**) of *Peziza fulgens* Pers. as the basic (type?) species of "*Peziza* Dill. ex. p., subgen. *Aleuria* Fr., Fuck., b. [sect.?] *Plicaria* Fuck. p. p." This will not serve as typification, however, since *P. fulgens* was not a member of the genus as originally erected by Fuckel.

The next typification known to me was by Boudier (**1885**), who restricted the genus to spherical-spored forms. He divided the genus into two groups (without indication of rank), and wrote: "Type des Levispores: *P. leiocarpa* Curr., et des Verrucispores: *P. trachycarpa* Curr." While neither of these species was among the 12 described by Fuckel in his flora, his original diagnosis closed with the statement: "Peziza trachycarpa Curr. in Rbh. F. eur. 620. et Peziza Marsupium Pers. Myc. eur. I. pag. 228. etiam hujus generis sunt." Boudier was thus clearly within his rights in selecting *Peziza trachycarpa* as a type for one subdivision of the genus (the type subdivision by inference, since *P. leio-*

carpa was not mentioned by Fuckel). This is as far as known the first valid selection of a lectotype for *Plicaria*.

In the work by Rehm (**1894**), all of the 14 species mentioned by Fuckel are treated. The spherical-spored ones are ranged in two genera, *Plicariella* (Sacc.) Rehm for the species with asci blueing in iodine and *Barlaea* Sacc. for the (one) spherical-spored species lacking this character. The oval-spored species he treated under *Plicaria* Fuckel emend. Rehm for the species with asci blueing in iodine (though he transferred one iodine-positive species to *Pustularia* Fuckel), with the (one) iodine-negative species referred to *Discina* (Fr.) Fr. It is this emendation of *Plicaria* which is followed in nearly all of the German literature. Though no type was designated by Rehm, he clearly intended to limit the genus to certain iodine-positive, oval-spored species.

At least two other species have been designated the type of *Plicaria*. Von Höhnel (**1918**) indicated *Plicaria carbonaria* Fuckel (≡ *Peziza anthracina* Cooke) as the type (spherical-spored, iodine-negative), presumably merely because it is the first species listed by Fuckel. Clements and Shear (**1931**) chose an oval-spored species, *Plicaria badia* (Pers. ex Mérat) Fuckel, as the type, following Rehm's concept of the genus, which may represent the first typification of *Plicaria* Fuckel emend. Rehm 1894 *non* emend. Boudier 1885.

It would appear that there are no conflicting earlier homonyms, and that the nomenclatural status of the genus *Plicaria* Fuckel emend. Boud. is valid, and that the genus is typified by the lectotype *Plicaria trachycarpa* (Curr.) Boud. Its taxonomic status, however, is open to question. For those taxonomists, e.g., Batra (**1961**), Le Gal (**1947**), etc., who would segregate a genus of spherical-spored, iodine-positive species, *Plicaria* is available and should replace the more recent names *Plicariella* and *Detonia* Sacc. which appear in some of the literature. If one does not recognize the iodine reaction as constituting a good taxonomic grouping, and would merge these species with nonreactors and *also* with species possessing carotenoid pigments, as does Seaver (**1928**), the generic name *Lamprospora* de Notaris would be older and should be used. Further, if spore shape is held not to be a significant generic character, as I believe to be the case among the iodine reactors, the generic name *Peziza* St. Amans may be applied (in my current classification *Peziza trachycarpa* falls in *Peziza* subgen. *Galactinia* Cooke).

The discomycete published by Schneider (**1954**) as *Plicaria fulva* is not uncommon in the greenhouses at Cornell University, both in the apothecial state and in the imperfect state, occurring on steam sterilized soil, pots, vermiculite, etc. I reported on the occurrence of both stages

("*Galactinia*" and "*Botrytis*-like") in a verbal paper at the annual meeting of the Mycological Society of America in 1948, but have ever since hesitated publishing my data since I was unable to match any type specimen with it. I still hesitate to propose any "new" species in a genus as large and as poorly worked as *Peziza,* to which I believe the fungus belongs. Now that Dr. Schneider has provided a name for the apothecial state, and after many years of examining type specimens, I can state that I still know of no other available name to apply to this discomycete. (Her description is excellent. Only in one feature need it be emended, since I invariably found the American material to have reticulate spores, while she described *Plicaria fulva* with "asperulate" spores; the specimen which she kindly lent me has reticulate spores. The markings are slight, and the reticulum is seen easily only when stained in heated cotton-blue dyes.)

The imperfect state is very characteristic, and is almost certainly the fungus described by Wolf (**1955**, **1957**) as *Mycotypha dichotoma.* It was merely recorded as "*Galactinia* sp. (*Botrytis* stage)" by Reese et al. (**1950**) from cultures I had sent. During my search for an appropriate generic name for the imperfect state the late Dr. S. C. Damon (*pers. comm.*) suggested it could be referred to the genus *Hyphelia* Fr. There is no doubt that the imperfect state is also very similar to that described for *Tomentella* Pers. ex Pat. by Brefeld (**1889**), and recalls the imperfect states of other members of the Thelephoraceae described by Maxwell (**1954**). These fungi lie somewhere near *Botrytis* "Pers.," *Rhinotrichum* Corda, and *Oedocephalum* Preuss. Dr. S. J. Hughes, of the Plant Research Institute, Ottawa, our current authority on the Hyphomycetes, has been most cooperative in working with me on a name for the imperfect state, and has suggested that it is best referred to the genus *Ostracoderma* Fr.

To my mind, Dr. Schneider's oval-spored species should be referred to the genus *Peziza,* subgenus *Galactinia,* but unfortunately her appropriate epithet cannot be transferred because of an earlier homonym. I have therefore chosen the generic name of the imperfect state for the new specific epithet: **Peziza ostracoderma** Korf, nom. nov. (basionym: *Plicaria fulva* Schneider, Zentralbl. Bakt. II **108**: 153. 1954, non *Peziza fulva* Micheli ex Pers., Myc. eur. **1**: 241. 1822). Stat. conid.: *Ostracoderma* sp. (= ? *Mycotypha dichotoma* Wolf).

It is of interest to note that another *Ostracoderma* has been described by Wolf (**1958**) as *Rhinotrichum trachycarpa,* and that this represents the conidial state of the spherical-spored *Peziza trachycarpa* (type of the genus *Plicaria*). The occurrence of similar *Ostracoderma* imperfect

states in the life cycles of two discomycetes which seem to differ fundamentally only in the shape of their ascospores lends additional weight to my taxonomic conclusion that the genera *Plicaria* and *Peziza* should be merged.

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NOTES AND BRIEF ARTICLES

Another Source of Mycotypha dichotoma

Examination of fungus colonies of unfamiliar appearance revealed *Mycotypha dichotoma* Wolf (Wolf, F. A. *Another Mycotypha.* Jour. Elisha Mitchell Sci. Soc. **71**: 213–217. 1955) growing on Sabouraud's and cornmeal agar plates inoculated with sputum. The morphology of the organism is distinctive and can be readily identified, even though the taxonomic position is unsettled. In 1957, Wolf (Wolf, F. A. Is *Mycotypha* a Phycomycete? Mycologia **49**: 280–282. 1957) questioned the assignment of this organism to the phycomycetous fungi and described it as an unnamed form genus among the Moniliaceae.

To my knowledge, all previous isolations of the fungus have been made from greenhouse soil. Our patient denied any contact with soil, either in a greenhouse or out-of-doors. It would seem this fungus must be added to the long list of organisms called air-borne contaminants by the medical mycologists.—Shirley McMillen, Mycology Laboratory, Hektoen Institute for Medical Research of Cook County Hospital, Chicago, Illinois.

Notes on the Flagellation of Zoospores of Aphanomyces euteiches

An accepted characteristic of truly biflagellate Phycomycetes is that their motile spores have one whip-lash type and one tinsel type flagellum. Although this is the case in apparently all species whose flagella have been studied in detail, there are many species whose flagella have not been studied to such a degree. Apparently the details of flagellation in *Aphanomyces euteiches* Drechs. have not been published.

A zoospore suspension was prepared from mycelial mats grown 4 days at 24° C in maltose-peptone broth (3.0 g maltose and 1.0 g peptone in 1 liter of distilled water). Zoospore formation was induced by replacing the medium with an equal volume of sterile tap water, and replacing this water 2 hours later with enough sterile distilled water to cover the mats. Active zoospores were abundant 12 hours later.

Zoospores were prepared for microscopical study using methods similar to those of Couch (**1**), Ellison (**2**), and Koch (**4**). A drop of

the zoospore suspension was placed on each of several slides, inverted immediately over a vial of 2% aqueous osmium tetroxide for 45 seconds, and then placed in a desiccator until dry. A drop of Loffler's mordant [1] was then added to each preparation and heated for 1 minute being careful not to let the mordant boil. The slides were dipped in distilled water to remove the mordant, and Loffler's flagella stain [2] was added for 2 minutes, and removed by dipping in distilled water. The preparations were then dried thoroughly in air and mounted in balsam.

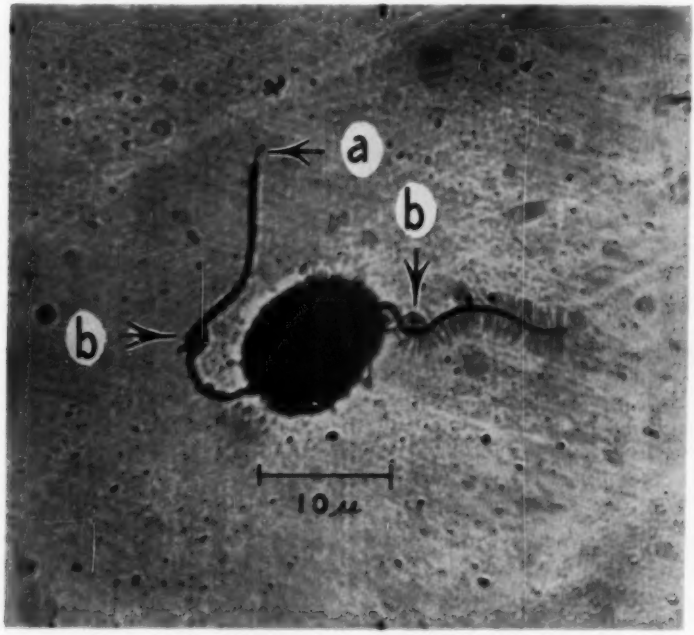

FIG. 1. Zoospore of *Aphanomyces cuteiches*.

The accompanying photomicrograph illustrates the whip-lash and tinsel flagella found on zoospores of *A. euteiches* (FIG. 1). The whip (a) is visible at the very tip of the flagellum on the left. The flagellum on the right shows about 80 tinsels along its entire visible length. Note also the "bubble" (b) in the crook of each flagellum, but especially

[1] Loffler's mordant: 10 parts 20% aqueous tannic acid, 5 parts saturated aqueous ferrous sulphate, and 1 part saturated alcoholic fuchsin.

[2] Loffler's flagella stain: 4% gentian violet in anilin water plus 0.1% sodium hydrate. This stain must be used within 2 weeks.

noticeable in the left flagellum. Similar phenomena were described by Ferris (3) as occurring on zoospores of *Phytophthora infestans* (Mont.) deBary and illustrated by Koch (4) as occurring in *Catenochytridium carolinianum* Berdan and a species of *Chytridium*. Koch and Ferris reported these "bubbles" occurring as the flagella are being withdrawn. Manton et al. (5) considered them the result of gathering of the flagellar sheath as it is being stripped off. Occasionally "paddles" (rolling of flagella) were observed on the tips of the flagella of *A. cuteiches* zoospores as illustrated by Ferris in zoospores of *P. infestans*.—JOHN L. CUNNINGHAM AND D. J. HAGEDORN, Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin.

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AQUATIC HYPHOMYCETES FROM WYOMING

During the period from July 21 to September 3, 1959, collections of aquatic hyphomycetes were obtained from streams in Albany County, Wyoming and the Jackson Hole area of northwestern Wyoming. These fungi were found growing on submerged, decaying leaves of *Salix* spp., *Populus* spp., *Betula glandulosa* Michx. and *Alnus tenuifolia* Nutt. Twelve species, representing nine genera, were identified. This appears to be the first report of the occurrence of these fungi in the Rocky Mountain region. This survey was part of a project supported by a grant from the New York Zoological Society. The writer acknowledges the privilege of working at the Jackson Hole Biological Research Station, Dr. L. Floyd Clarke, Director. The following species were collected:

Tetracladium marchalianum de Wild.

Teton County: Spring Creek, Granite Creek, Arizona Creek, Pacific Creek; Albany County: Rock Creek north of Laramie, S. Fork of Pole Creek east of Laramie.

Lemonniera aquatica de Wild.
Teton County: Spring Creek, Granite Creek, Pacific Creek; Albany County: S. Fork of Pole Creek.
Lemonniera brachycladia Ing.
Albany County: S. Fork of Pole Creek.
Clavariopsis aquatica de Wild.
Teton County: Spring Creek, Pacific Creek; Albany County: S. Fork of Pole Creek, Sybille Creek.
Heliscus aquaticus Ing.
Teton County: Spring Creek.
Articulospora tetracladia Ing.
Teton County: Spring Creek, Arizona Creek, Pacific Creek.
Anguillospora crassa Ing.
Teton County: Spring Creek.
Anguillospora longissima (Sacc. & Syd.) Ing.
Teton County: Spring Creek; Albany County: S. Fork of Pole Creek.
Tetrachaetum elegans Ing.
Albany County: S. Fork of Pole Creek.
Tricladium splendens Ing.
Teton County: Spring Creek.
Tricladium angulatum Ing.
Albany County: Rock Creek, north of Laramie.
Margaritispora aquatica Ing.
Teton County: Spring Creek, Pacific Creek.

JOHN W. BAXTER, University of Wisconsin, Milwaukee.

ENDOGENOUS CONIDIUM PRODUCTION IN PESTALOTIA

Even though conidia of imperfect fungi generally seem to be cut off from the parent plant by constriction, the production of reproductive cells from within the hyphae of fungi is relatively common. Some fungi, as *Chalara*, are generally recognized as having endogenous conidia, and may be keyed by the use of that characteristic (Clements and Shear, **1931**; Barnett, **1955**). Emergence of conidia from hyphal tips has been described for *Clonostachys*, *Gliocladium*, and *Allescheria* (Pinkerton, **1936**), *Penicillium* (Scaramella, **1928**), *Cadophora* (Melin and Nannfeldt, **1934**), *Ceratostomella* (Dade, **1928**), and many others.

The characteristically appendaged, multicellular conidia of *Pestalotia* are rather complex reproductive bodies, but they have, at least in the

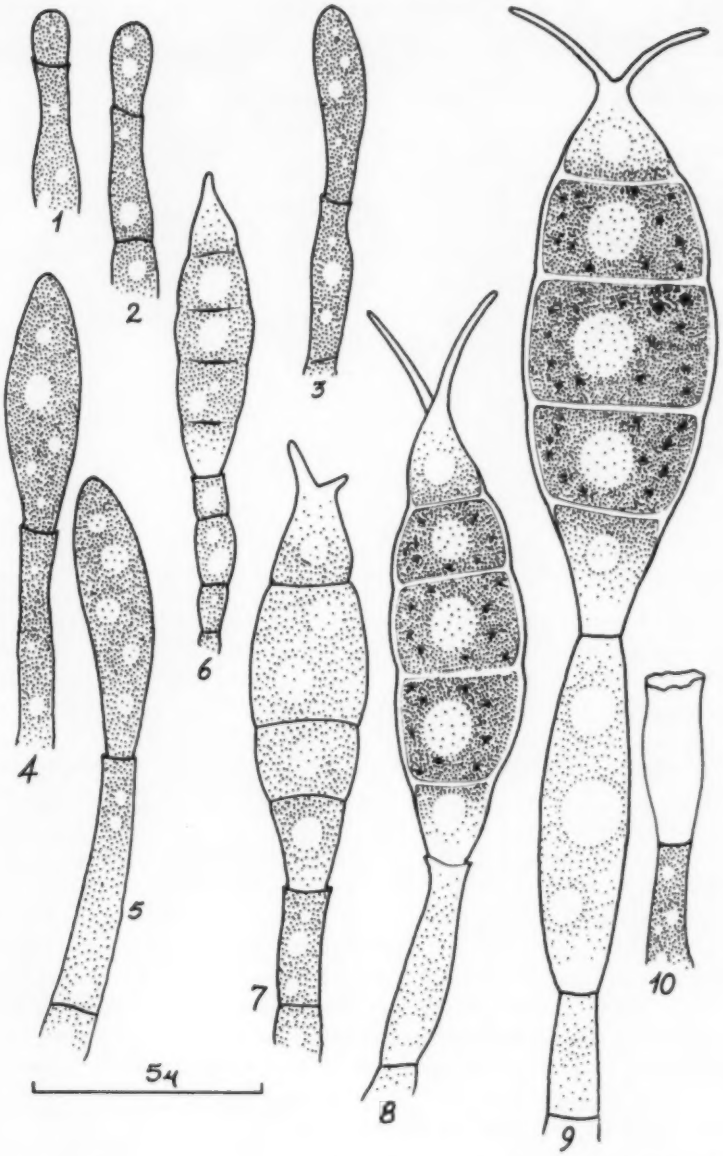
strain reported here, an endogenous origin, being forced out from ruptured hyphal tips in an incomplete state and undergoing maturation while still attached. Neither Leininger (**1911**) nor Klebahn (**1913**), who made extensive observations on the genus, described this method.

The *Pestalotia* from which the accompanying figures were made is very similar to *P. aletridis* (Pat.) Guba. It was isolated from yellow pine panels which had been submerged for eight months in the salt water of Limon Bay, Panama, and was cultivated on natural sea-water agar containing 0.5% glucose and 0.1% Difco yeast extract. The figures were made from living material.

The first indication that the constricted tip of a hypha is about to produce a conidium is the rupture of the end, followed by the outward swelling of the protoplast. The end of the old cell wall is visible as a tightly fitting but distinct collar around the bulging apex (FIG. 1). In *P. palmarum* (Leininger, **1911**), conidia mostly arise laterally as evaginations which are later cut off by cross walls. That organism differs from *P. aletridis* also in the sequence of events in enlargement, septation, and production of setae.

In *P. aletridis,* vacuoles move into the incipient conidium as it increases in length and diameter (FIGS. 2–4). The presence of five cells in the mature conidium is foreshadowed by the frequent appearance of five vacuoles some time before the dividing walls start to form (FIG. 5). Once the conidium begins to assume its typical shape, four septa are laid down, cutting the body into its characteristically five-celled condition. The ultimate cell of the conidium produces first one (FIG. 6) and then a second terminal appendage, the distal setae (FIG. 7). Two is normal in *P. aletridis,* though three appear rarely. The setae achieve their maximum length before the conidium reaches its full size (FIG. 8). The proximal and the terminal cells remain hyaline, while the three middle cells fill with densely granular and vacuolate protoplasm and darken, building walls which are thicker than those of the end cells (FIG. 9). The middle cells are the ones which normally function when the conidium germinates.

Before a hypha releases a conidium, the basal conidial cell pulls in from its surrounding hyphal wall and the shrunken tip becomes the slender so-called pedicel of the typical *Pestalotia* conidium. When the conidium is liberated, the empty hyphal cell is left behind (FIG. 10). It apparently does not proliferate, each of the numerous conidia in a pycnidium being produced at the apex of a separate hyphal branch.—DON RITCHIE, Department of Botany, Barnard College, Columbia University, New York *27*, N. Y.



FIGS. 1–10. Development of endoconidia by *Pestalotia alctridis*. FIGS. 1–9, early stages, from incipient conidium at hyphal tip in FIG. 1, to mature, multicellular conidium just before release in FIG. 9. FIG. 10, empty terminal cell of hypha after release of conidium.

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REDUCING DESICCATION AND MITE INFESTATION OF CULTURE MEDIA [1]

The desiccation of agar media in flasks and culture tubes is a problem in many laboratories, particularly in arid and semiarid regions. Conventional means of controlling such evaporation have included using screw-capped tubes and vials, keeping tubes inside of screw-capped fruit jars (1), and capping tubes and flasks with various nonpermeable materials.

A method of reducing desiccation that has certain advantages for large volume work has been tried successfully at the North Dakota Agricultural Experiment Station at Fargo. Polyethylene plastic bags are placed over the containers of media and sealed with rubber bands or greenhouse plant ties (Twistems). By using this technique, it is possible to seal flasks and baskets of test tube slants and store them for long periods under average conditions. Potato-dextrose agar slants and flasks maintained in this manner on laboratory shelves at room temperature (about 23° C) have remained suitable for culturing more than 10 months after preparation, the length of tests to date. Fungus cultures grown upon this stored medium appear similar to cultures grown on freshly prepared material. Comparable unsealed agar preparations were dried beyond use after less than one month of storage (FIG. 1).

[1] Published with the approval of the Director, Research Paper No. 12, North Dakota Agricultural Experiment Station.

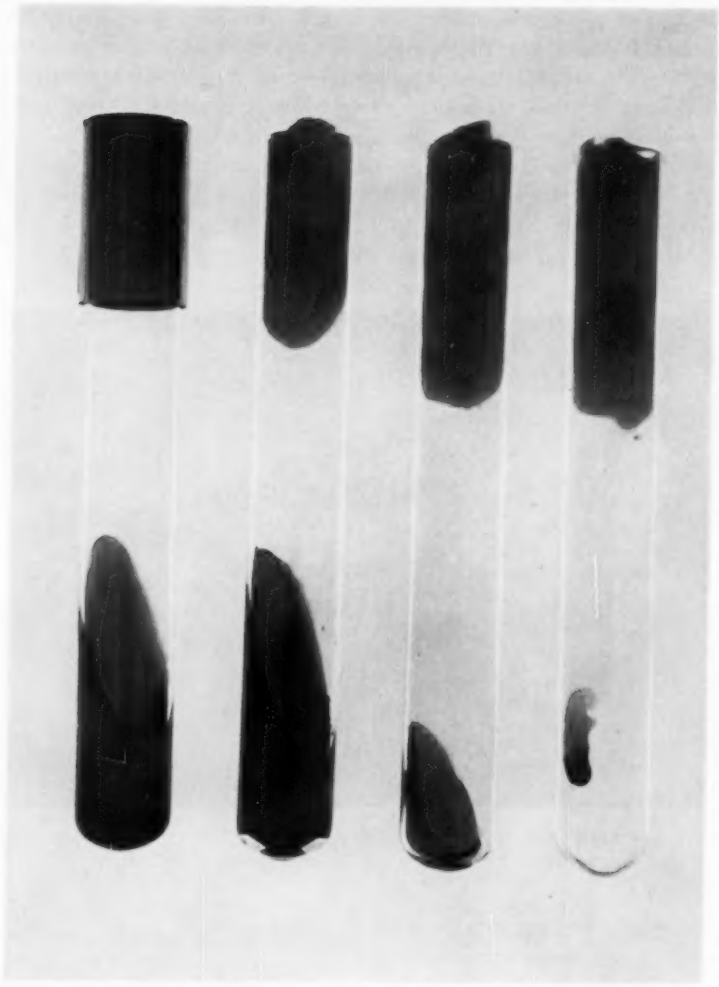

FIG. 1. The 2 left tubes were sealed in plastic for 10 months, while the right center and right tubes were kept unsealed for 1 and 3 months, respectively. All tubes were held at room temperature, and dye was added to the agar for photographic contrast. The left tube demonstrates a stainless steel closure mentioned in the text.

The main value of this procedure has been the ease of preparing large quantities of agar media at one time for routine culture work so that with sufficient storage area, media has to be prepared only once in several months. The method found satisfactory has been to sterilize many flasks and baskets of tubes, allow them to dry overnight, and seal them within sacks which may be opened individually as needed (FIG. 2).

The high humidity within the sacks might favor growth of contaminants on cotton plugs, as reported by Eddins (1) in screw-capped jars. No evidence of this has been observed but if the problem should arise, the use of stainless steel closures should correct it (2) (FIG. 1). In

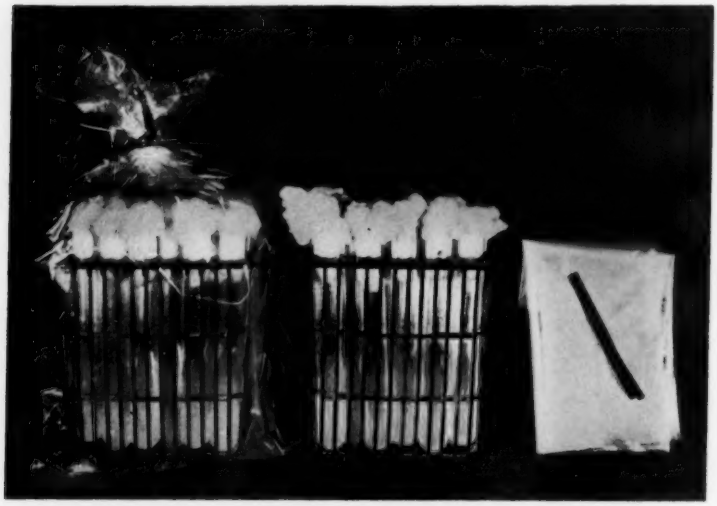

FIG. 2. An illustration of a plant tie and plastic bag on the right, a basket of tubes in the center, and a sealed basket of tubes on the left.

cases where nutrient deterioration is a critical factor, the media may be kept in a cool room or refrigerator.

In addition to preserving sterile media, fungus cultures of 2 species, *Helminthosporium sativum* Pam., King and Bakke (*Helminthosporium sorokinianum* Sacc.) and *Septoria passerinii* Sacc., have remained moist and viable for over 6 months between transfers when sealed in plastic bags and stored in a refrigerator (5° C). Baskets of cultures can be maintained in this method, so that it is not necessary to seal individual tubes. Also, the air-tight bags appear to prevent the introduction of

mites into enclosed cultures, although a lack of serious mite infestations at Fargo has prevented full substantiation of this point. Sealing cultures would not be advisable where continual aeration is desired.—DONALD J. MORTON, Department of Plant Pathology, North Dakota State College, Fargo.

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BASIDIOMYCETES ISOLATED FROM SOIL

Basidiomycetes are frequently found fruiting on soil in both field and woodland, yet they are seldom reported in lists of fungi isolated from soils. Gilman (**1957**) lists only a single species in his *Manual of the Soil Fungi,* and a review of recently published lists (Cooke, **1959**; Domsch, **1959**; Sewell, **1959**; Al-Doory et al., **1959**; Butler and Hine, **1958**; Miller et al., **1957**) shows clearly how infrequently these fungi are isolated in studies of soil fungi. Warcup (**1959**) has amply emphasized the difficulty of isolating basidiomycetes by the conventional techniques of soil mycology. Sterile mycelia, identified as basidiomycetes by the presence of clamp connections or by other criteria (Warcup, **1959**) are occasionally reported (Sewell, **1959**), but these only rarely produce fructifications.

During investigations of the soil mycobiota carried out over the past 5 years, I have succeeded in isolating from soil 4 basidiomycetes which were induced to sporulate in culture. In view of the relative infrequency with which sporulating basidiomycetes are reported in studies of soil fungi, the isolation of these 4 species is here reported.

A brown-spored agaric, identified as *Pholiota marginata* (Fr.) Quél. was isolated by dilution plate methods in the spring of 1954 from a soil sample collected in an oak-hickory forest near Iowa City, Iowa. Typical sporocarps were formed on the original isolation medium and in subsequent transfers. The fungus has been carried in active culture since its isolation and fruits readily on simple media.

A fungus identified as *Irpex lacteus* (Fr.) Fr. was isolated by the soil plate technique (Warcup, **1950**), also from oak-hickory forest soil collected near Iowa City. In culture the fungus produced a resupinate

toothed hymenium, typical of the family Hydnaceae, bearing cystidia, basidia and spores.

Schizophyllum commune Fr. was isolated by dilution plate techniques in 1958 from Honduran soil, during a study of banana rhizosphere fungi. It was obtained from both rhizosphere soil and macerated root tissue, and constituted a high percentage of the isolates from a single root sample. In culture, these isolates displayed a variety of morphological forms, similar to those illustrated by Raper and Krongelb (**1958**). Miller et al. (**1957**) recently reported the isolation of this species from cultivated soils in Georgia.

The fourth basidiomycete, a species of *Pellicularia,* was isolated from Honduran banana soil, using a Mueller and Durrell (**1957**) soil immersion tube containing a rose bengal-streptomycin medium. This isolate produced only a sterile mycelium when subcultured on a variety of media, but sporulation was induced by placing a block of mycelium on potato dextrose agar in sterile distilled water. Abundant basidia and spores, as well as globose conidia, were produced within a few days. *Pellicularia* is the only basidiomycete included in Gilman's *Manual of Soil Fungi.* It has also been reported by Miller et al. (**1957**) and by Warcup (**1959**).

My experience in isolating basidiomycetes from soil has been in complete accord with the numerous reports on the paucity of these fungi in studies of soil fungi. The results reported here do indicate that they may occasionally be isolated, and it is probable, as has been pointed out by Warcup (**1959**), that many of the sterile mycelia frequently isolated in soil studies actually represent monocaryotic basidiomycetes.

Interestingly, the 4 species recorded here were isolated from samples collected at a depth of 10–12 inches. Their isolation from this depth may indicate that it was at this level that these fungi were active, but it is probable that they were more readily isolated from the deeper soil layers because of the lesser number of total fungi present.

A portion of this work was done in the Mycological Laboratory of the State University of Iowa under the supervision of Professor G. W. Martin, whom I wish to thank for identifying *Pholiota marginata* and *Irpex lacteus.*—ROGER D. GOOS, Central Research Laboratories, United Fruit Company, Norwood, Massachusetts.

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AN EXTREMELY TOLERANT YEAST FROM HUMAN CADAVERS

It recently occurred to me, while discussing tolerance of fungi to unfavorable environments with a group of my graduate students, that other mycologists would be interested in knowing of a yeast which we found in human cadavers here at the University of Missouri some six or seven years ago. Hence this short note. I understand from my anatomist acquaintances that such occurrence of a microorganism is not unique in cadavers, but that it is unusual. To my knowledge, no such organism has previously been isolated and reported.

In 1954, members of the Department of Anatomy of the School of Medicine, University of Missouri, were plagued by an infestation of a slimy growth on the interior of their teaching cadavers. Many cadavers, when opened for dissection, were infested so heavily that mesenteries and fragile tissues were nearly disintegrated. The livers, generally, were heavily infested, often being completely white with the growth, but any of the organs and tissues appeared to be susceptible to infestation by the organism.

Obviously, this presented something of a problem to the anatomy

people in their teaching program and a friend on the anatomy staff turned to me for suggestions of control. Initially pure cultures were readily obtained by streaking bits of infested lung directly onto ordinary potato dextrose agar. I easily identified the organism as a yeast, but could not identify it further immediately. The anatomy staff, naturally, was in something of a hurry for advice and so I sent a culture of the yeast to Dr. Lynferd Wickerham, NRRL at Peoria, Illinois, for assistance in identification and in recommendations for control. Unfortunately for the Anatomy Department, neither Dr. Wickerham nor I were the least bit useful in the solution of the problem. The problem sporadically dissipated and reoccurred for two years as the anatomy people threw various chemicals into, and onto, their cadavers, and finally, was solved when the Medical School moved into completely new quarters with new cadavers, new storage vats, and new preservatives.

Of course, Dr. Wickerham did identify the yeast. It proved to be a previously undescribed species of his favorite genus, *Hansenula*. He is presently working with this yeast and tells me that he finds it quite interesting. He has made other isolates of the same species from pieces of tissues from cadavers which I have sent him. He is using these isolates in studies of the organism.

I am impressed by the tolerance exhibited by this organism to the horrid chemicals in which cadavers are embalmed. The embalming fluid used was composed of 2000 ml glycerin, 2000 ml water, 1950 ml alcohol, 230 ml phenol (saturated solution), 840 ml formalin (commercial 40% formaldehyde), 180 ml furfural (commercial 100%), and 50 g thymol (added as crystalline material). 7250 ml of this solution was introduced under pressure into the femoral artery of each cadaver so that it permeated well into the tissues. The cadavers were stored in tanks containing a 3–4% solution of phenol. When we were unable to suggest methods of control, the persons in charge of the cadavers made it a practice to paint anything within reach directly onto the affected portions of the cadavers as soon as growth appeared. Thymol, sodium azide and thiolutin were used in this fashion to no avail. Zephirin chloride finally gave some measure of sporadic control of the infestation.

Dr. Wickerham tells me that his preliminary studies have shown that this cadaver yeast is truly an extremely tolerant organism. We may hope to hear from him concerning both the tolerance and the sexuality of this *Hansenula* sp. isolated from a rather unusual habitat.—JOHN E. PETERSON, Department of Botany, University of Missouri, Columbia.

NEW SPECIES OF DISCOMYCETES FROM INDIA—II

The new species described below were partly published as "Taxonomic Species" in English only (Batra, **1960**). The "Taxonomic Species VIII" (p. 30 *loc. cit.*) is now named as *Lamprospora multiguttula*, "*L. choprae*" (p. 30) as *L. chopraiana*, "*Plicaria* Taxonomic Species VI" (p. 29) as *P. papillosa*, and "*P. pseudoplanchonis* in herb." (p. 29) as *P. pseudoplanchonis*.

Colors in quotation marks are taken from Ridgway (**1912**). The type specimens have been deposited in the Department of Plant Pathology Herbarium (CUP–I), Cornell University, Ithaca, New York.

Lamprospora multiguttula Batra, sp. nov. (FIGS. 1–3)

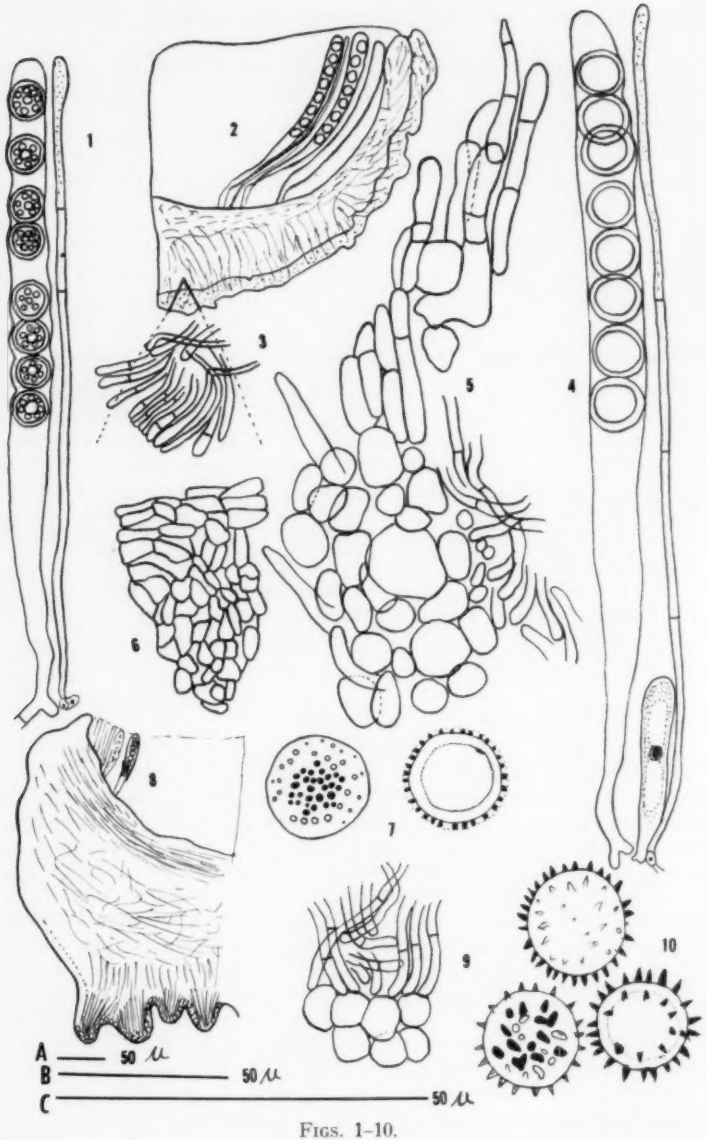
Apothecia gregaria, sessilia, fragilia; discus discoideus, 2–3 mm diam., "Orange Chrome" ad "Cadmium Orange"; apothecii secti hymenium 200–220 μ, aurantiacum; subhymenium inconspicuum, textura intricata, hyphis subhyalinis; excipuli medullaris textura intricata, 50–60 μ, hyphis 2–3 μ diam., excipulum exterius conspicuum, textura intricata compacta, 28–36 μ, plurimis hyphis intendentibus extra, 2–3 μ diam., apicibus usque ad 4 μ inflatis; asci operculati, cylindracei, octosporidei, jodo non coerulescentes, basim versus gradatim attenuati, 210–240(–290) × 14–19 μ; ascosporae perfecte globosae, monostichae, hyalinae, leves, 5–9-guttulatae, sporidiis 12–13(–14) μ; paraphyses non ramosae, septatae, auranticae, ad apicem crassatae, 200–240 × 2–3 μ, ad apicem ad 4 μ; habitatio super terram; locus typi Mussoorie, Indiae Orientalis; typus CUP–I 104; etymologice "multiguttula" e Latino; *multi* = many, *guttula* = a small drop.

Lamprospora chopraiana Batra, sp. nov. (FIGS. 4, 5)

Apothecia sparsa, sessilia, fragilia; discus cupulatus vel discoideus, margine minute crenulata, 2–4 mm diam., 1–1.5 mm alt., intus mutans e "Sanford Brown" ad "Amber Brown" ad "Madder Brown," rupti disci carne non mutante colorem; apothecii secti hymenium furfuraceum, 230–250 μ; subhymenii et excipuli medullaris textura intricata, excipulis cunctis 120–160 μ, hyphis 4–5 μ diam., subhyalinis; excipulum exterius 80–120 μ, textura globulosa, quisdam cellulis externis elongatis, pilosis, cellulis internis globosis, 15–30 × 10–25 μ; asci operculati, cylindracei, octosporidei, ad basim subito attenuati, jodo non coerulescentes, 200–240 × 15–18 μ; ascosporae perfecte globosae, monostichae, hyalinae, leves, uniguttulatae, (11–)14–17 μ diam.; paraphyses non ramosae, septatae, furfuraceae, 190–250 × 3–4 μ, ad apicem usque ad 5 μ; habitatio super terram humorosam inter folia *Cupressi* spp.; locus typi Mussoorie, Indiae Orientalis; typus CUP–I 73, August 4, 1952; etymologice *chopraiana* honorans Professorem Ram Saran Chopra, Department of Botany, Panjab University, India.

Plicaria papillosa Batra, sp. nov. (FIGS. 6, 7)

Apothecia sparsa, sessilia, fragilia; discus cupulatus, margine crenata, 5–10 mm diam., usque ad 5 mm alt., extra badius et papillosus, papillis 150–300(–400) μ alt., 120–250 μ lat. ad basim; apothecii secti hymenium castaneum vel purpureum, 370–



FIGS. 1–10.

FIGS. 1–3. *Lamprospora multiguttula.* FIG. 1. An ascus and a paraphysis, × 500. FIG. 2. T. S. of an apothecium showing hymenium, subhymenium, medullary excipulum and ectal excipulum, × 130. FIG. 3. Ectal excipulum, × 500. FIGS. 4, 5.

400 μ; subhymenii textura intricata inconspicua; excipuli exterioris et excipuli medullaris textura angularis, excipulis cunctis 311–410 μ, cellulis 25–43 × 11–18(–30) μ; asci operculati, cylindracei, octosporidei, ad basim gradatim attenuati, jodo coerulescentes, 360–380 × 19–21 μ; ascosporae perfecte globosae, fulvae, uniguttulatae, verrucosae, 17–18(–20) μ diam.; paraphyses non ramosae, septatae, brunneae, 350–390 × 4–6 μ, ad apicem usque ad 11 μ; habitatio super terram; locus typi Dalhousie, Indiae Orientalis; typus CUP–I 72, July 15, 1955; etymologice ex Latino, *papilla* = nipple.

Plicaria pseudoplanchonis Batra, sp. nov. (FIGS. 8–10)

Apothecia gregaria vel sparsa, subsessilia, fragilia; discus cupulatus vel subdiscoideus, margine crenata, 1–1.2 cm diam., 5–8 mm alt., extus purpureus et tuberculatus vel papillosus, papillis minoribus, 36–100 × 21–36 μ; apothecii secti hymenium purpureum, 350–380 μ; subhymenium inconspicuum, textura intricata, hyphis brunneis; excipulum medullare 300–410(–450) μ, textura intricata, plurimis hyphis intendentibus extra, 1–2 μ diam.; excipulum exterius 32–40 μ, textura globulosa, cellulis 8–18 × 7–11 μ; asci operculati, cylindracei, octosporidei, ad basim gradatim attenuati, jodo coerulescentes, 316–360 × 16–20 μ; ascosporae perfecte globosae, fulvae, spinosae, spinis 2–3 μ alt., 1.5 μ lat. ad basim munitae, uniguttulatae, (13–)15–18 μ spinis inclusis; paraphyses non ramosae, septatae, brunneae, 350–400 × 3–4 μ, ad apicem usque ad 5 μ inflatae; habitatio super terram; locus typi Mussoorie, Indiae Orientalis; typus CUP–I 71, August 16, 1952; etymologice e Graeco, *pseudos* = false, *planchonis* ex *Lamprospora planchonis* (Dun.) Seaver.

This study was partly conducted at Swarthmore College, and has been supported by American Philosophical Society (Grant no. 325—Johnson Fund) and by Grant no. G-9041 from the National Science Foundation to Dr. Richard P. Korf, Cornell University, "Discomycete Flora of Asia."—LEKH R. BATRA, University of Kansas, Lawrence, Kansas.

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Lamprospora chopraiana. FIG. 4. An ascus with ascospores and a paraphysis, × 500. FIG. 5. T. S. of an apothecium showing apothecial margin, ectal excipulum and a part of medullary excipulum, × 500. FIGS. 6, 7. *Plicaria papillosa.* FIG. 6. T. S. of an apothecium showing a papilla of ectal excipulum and a part of medullary excipulum which is indistinguishable from the former except for the direction of the cells, × 500. FIG. 7. Ascospores, × 1100. FIGS. 8–10. *Plicaria pseudoplanchonis.* FIG. 8. T. S. of an apothecium showing general outline of various tissues, × 130. FIG. 9. Ectal excipulum and a part of medullary excipulum, × 500. FIG. 10. Ascospores, × 1100. Scale A for FIG. 2, 8; scale B for the FIGS. 1, 3, 6, 9; scale C for FIGS. 7, 10. All the figures are drawn with the aid of a camera lucida.

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